K Arai, M. Tanaka, S. Yamamoto, M. Shimomura	Effect of pore size of honeycomb films on the morphology, adhesion and cytoskeletal organization of cardiac myocytes, Colloids and Surfaces A	Physicochemical and Engineering	313-314	530-535	2008
Y. Fukuhira, H. Kaneko, M. Yamaga, M. Tanaka, S. Yamamoto, M. Shimomura	Effect of honeycomb-patterned structure on chondrocyte behavior in vitro, Colloids and Surfaces A	Physicochemical and Engineering	313-314	520-525	2008
A. Tsuruma, M. Tanaka, S. Yamamoto, M. Shimomura	Control of neural stem cell differentiation on honeycomb films, Colloids and Surfaces A	Physicochemical and Engineering	313-314	536-540	2008
Daisuke Ishii, Hiroshi Yabu, Masatsugu Shimomura	Selective metal deposition in hydrophobic porous cavities of self-organized honeycomb-patterned polymer films by all-wet electroless plating, Colloids and Surfaces A	Physicochemical and Engineering	313-314	590-594	2008
Y. M. Chen, M. Tanaka, JP Gong, K Yasuda, S. Yamamoto, M. Shimomura, Y. Osada	Tuning of cell proliferation on tough gels by critical charge effect	J. Biomed. Mater. Res. A			in press

Y. Fukuhira, M. Ito, H. Kaneko, Y. Sumi, M. Tanaka, S. Yamamoto, M. Shimomura	Prevention of postoperative adhesions by honeycomb-patterned poly(lactide)film in rat experimental model	J. Biomed. Mater. Res.			in press
T.Okajima, M.Tanaka, S.Tsukiyama, T.Kadowaki, S.Yamamoto, M. Shimomura, H.Tokumoto	Stress Relaxation and Fluctuation of Living Cells Measured by Atomic Force Microscopy	Jp. J. Appl. Phys.			in press
T. Hayashi, M. Tanaka, S. Yamamoto, M. Shimomura, M. Hara	Direct observation of biocompatibility of PMEA(poly(2-methoxyethyl acrylate)) using Atomic Force Microscopy	Biointerphase			in press

Ⅳ．研究成果の刊行物・別冊

cases of BSL have been associated with the A8344G mutation of mitochondrial DNA.[5] The highest incidence has been reported in middle-aged men from Mediterranean countries with a male to female ratio of 15 : 1. A history of alcohol abuse is frequent although not always present. It is important to look for associated endocrine and metabolic disorders such as abnormal glucose tolerance, hypertriglyceridaemia, hyperuricaemia and renal tubular acidosis which are commonly associated in these patients.[1,6–8]

Cosmetic deformities, with associated psychological problems in severe cases, interference with neck movements and mediastinal involvement with secondary respiratory and digestive compromise, represent the main reasons for treatment in these patients.

Treatment for multiple symmetrical lipomatosis is palliative. Conservative therapeutic measures have been reported but at present lipectomy and liposuction, separately or in combination, are the only effective, although not definitive, therapeutic approaches. The choice of surgical technique must be individualized in each case and depends on diverse factors such as the medical antecedents of the patient and the location, extension and consistency of the pathological adipose tissue. Liposuction seems to be preferable in patients with soft localized fatty tumours of limited size, while lipectomy is indicated in larger defects and cases with infiltration of the mediastinum. Nonetheless treatment is not definitive and recurrence frequently occurs.[9,10]

We describe a case of BSL which shares some clinical aspects with HAART-related lipodystrophy and Barraquer–Simons disease. Our patient presented with atrophy of subcutaneous fat in the facial region and central distribution of adipose tissue in the upper back and abdominal region without systemic manifestations. These entities are associated with metabolic disorders, including hyperlipidaemia and insulin resistance. Mitochondrial defects have been related to the development of both BSL and HAART-related lipodystrophy. This similarity suggests the possibility of a common origin of these syndromes and that they represent different parts of the same spectrum.

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References

1 Debén Sánchez M, Veloso Rosendo M, Pérez Álvarez C, Pita Caaveiro L. Lipomatosis simétrica multiple: presentación de dos formas clínicas diferentes y revisión de la literatura. *An Med Interna* 2003; **20**:243–6.
2 Vargas-Díez E, Daudén E, Jones-Caballero M, García-Díez A. Madelung's disease involving the tongue. *J Am Acad Dermatol* 2000; **42**:511–13.
3 Fernández-Vozmediano J, Armario Hita J. Benign symmetric lipomatosis (Launois–Bensaude syndrome). *Int J Dermatol* 2005; **44**:236–7.
4 Ferrarini A, Milani E, Bottigelli M *et al.* Two new cases of Barraquer–Simons syndrome. *Am J Med Genet A* 2004; **126**:427–9.
5 Gámez J, Playán A, Andreu AL *et al.* Familial multiple symmetric lipomatosis associated with the A8344G mutation of mitochondrial DNA. *Neurology* 1998; **51**:258–60.
6 Morelli F, Feliciani C, De Benedetto A *et al.* Alcoholism as a trigger of multiple symmetric lipomatosis. *J Eur Acad Dermatol Venereol* 2003; **17**:367–9.
7 Saiz Hervás E, Martín Llorens M, López Álvarez J. Peripheral neuropathy as the first manifestation of Madelung's disease. *Br J Dermatol* 2000; **143**:684–6.
8 Brinkman K, Smeitink JA, Romijn JA, Reiss P. Mitochondrial toxicity induced by nucleoside-analogue reverse-transcriptase inhibitors is a key factor in the pathogenesis of antiretroviral-therapy-related lipodystrophy. *Lancet* 1999; **354**:1112–15.
9 Fischer M, Wohlrab J, Taube KM, Marsch WC. Intralesional injection of enoxaparin in benign symmetrical lipomatosis: an alternative to surgery? *Br J Dermatol* 1997; **137**:581–6.
10 Nakahigashi Y, Ogawa M, Kurokawa I, Kusumoto K. A case of benign symmetrical lipomatosis: histopathology and surgical treatment. *J Dermatolog Treat* 2000; **11**:201–5.

Key words: *Barraquer–Simons disease, benign symmetrical lipomatosis, HAART-related lipodystrophy, Launois–Bensaude syndrome*

Conflicts of interest: none declared.

Congenital ichthyosiform erythroderma mimicking ichthyosis bullosa of Siemens

DOI: 10.1111/j.1365-2133.2007.08244.x

SIR, Bullous congenital ichthyosiform erythroderma (BCIE) is a severe autosomal dominant inherited skin disorder caused by keratin 1 (K1) gene (*KRT1*) or keratin 10 (K10) gene (*KRT10*) mutations.[1,2] Patients with BCIE show generalized erythema and bullae from birth. Histopathologically, granular degeneration is seen in the middle to upper epidermis. Keratin clumps predominantly comprising K1 and K10 are observed in the spinous and granular layers.[3]

Ichthyosis bullosa of Siemens (IBS) is a relatively mild ichthyosis with superficial blister formation caused by keratin 2e (K2e) gene (*KRT2E*) mutations and in which granular degeneration is restricted to the uppermost spinous and granular layers.[4–6] Patients with IBS who had severe skin phenotypes and who had previously been misdiagnosed with BCIE from their clinicopathological findings were correctly diagnosed by molecular genetic testing methods.[7] Conversely, here we report a family with mild BCIE showing clinical and histological features similar to IBS. A heterozygous missense mutation p.Glu478Asp in *KRT1* was detected in this family.

The proband was a Japanese girl born to nonconsanguineous parents. The patient had no siblings, although her mother had a spontaneous abortion of a foetus after the birth of the

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proband. The proband's mother (patient 2), late grandfather (mother's father) and two great uncles were affected with a similar skin disease. The patient was born with flaccid blisters from birth forming on a background of erythematous skin over her whole body (Fig. 1a,b). During the first few months, she had developed erosions after mild mechanical trauma (Fig. 1c,d). At the age of 8 months, mild hyperkeratosis was apparent on her cubital fossa, popliteal fossa, axillae and inguinal areas and thick, diffuse scales and hyperkeratosis developed on both palms and soles. Her hair, teeth and nails appeared normal. Physical and mental growth was normal.

Skin biopsy specimens taken from the trunk at birth showed a thickening of the stratum corneum and granular degeneration only in the uppermost spinous and granular layer cells (Fig. 2a). By electron microscopy, irregularly shaped keratin clumps and cytolysis were seen only in the uppermost spinous and granular layers of the epidermis (Fig. 2c).

Patient 2 (mother of patient 1) showed erythema and mild hyperkeratosis on her extremities and axillae (Fig. 1e,f) together with thick, diffuse palmoplantar keratoderma (PPK). Clinically apparent blister formation was rarely seen.

A skin biopsy obtained from a hyperkeratotic lesion on her trunk revealed orthohyperkeratosis and granular degeneration with acantholysis restricted to the uppermost spinous and granular layers (Fig. 2b). By electron microscopy, the keratin filament network was disrupted only in the uppermost keratinocytes of the spinous and granular layers. Irregularly shaped, clumped keratin filaments were seen in the uppermost spinous and granular layers (Fig. 2c).

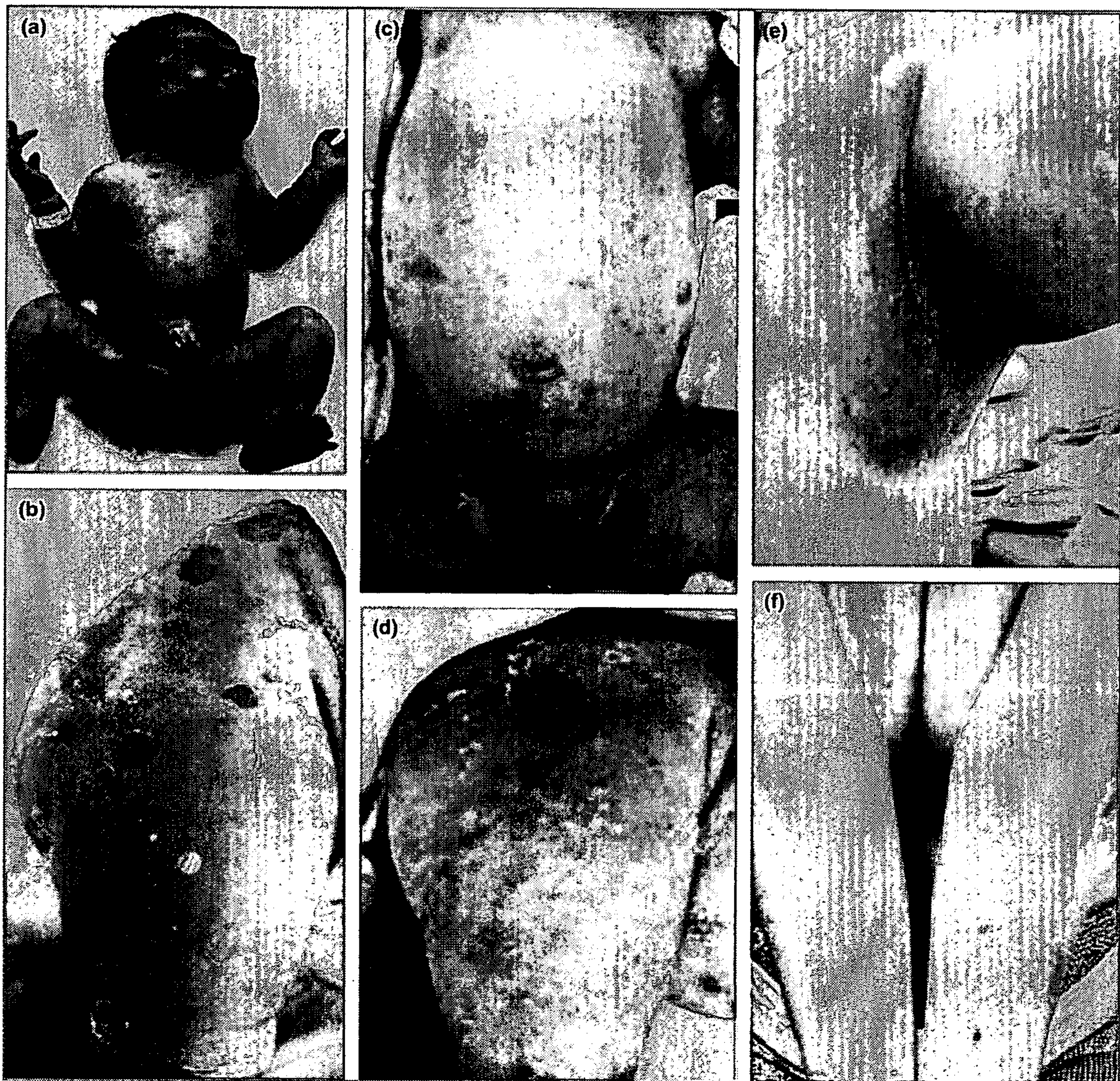

Fig 1. Clinical appearance. (a, b) Patient 1. At birth, superficial blisters and desquamation were seen on the erythrodermic skin. (c, d) Patient 1 at the age of 10 days; mild hyperkeratosis and erythema with superficial erosions were observed on some parts of the trunk and extremities including her inguinal areas and around the buttocks. (e, f) Patient 2. Hyperkeratosis was apparent in the axillae (e) and on the arms (f). Shallow erosions were also seen in the axillary regions (e).

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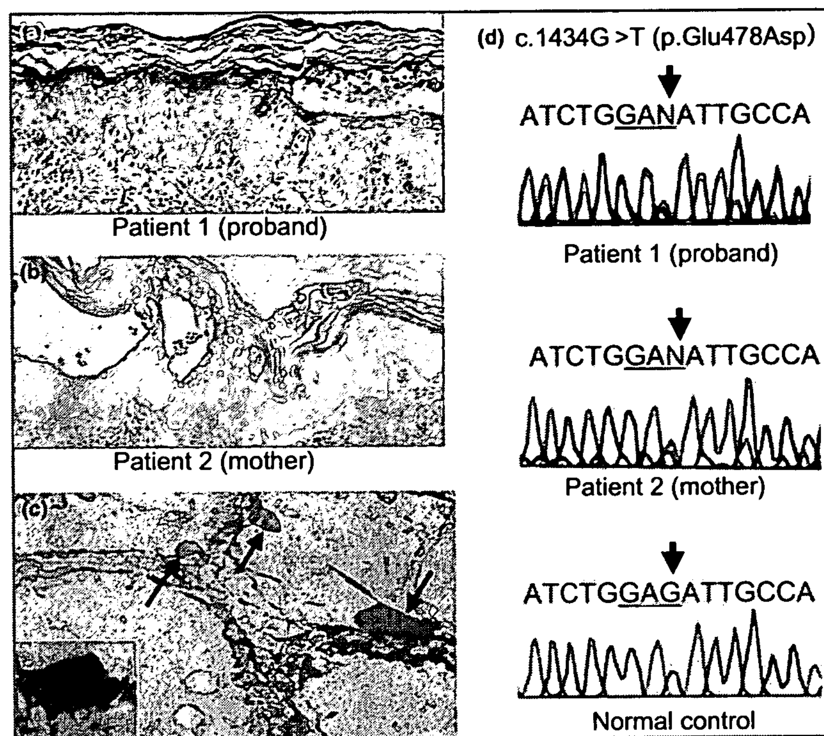

Fig 2. (a, b) Subcorneal blisters and granular degeneration were seen only in the uppermost spinous and granular layers in the hyperkeratotic skin lesions from patient 1 (a) and patient 2 (b). Original magnification × 30 (a, b). (c) Electron microscopy revealed irregularly shaped keratin clumps in the granular layer cells that showed vacuolar degeneration in patient 1. Inset: high-power view of clumped keratin filaments in patient 2. Original magnification × 10 000 (inset, × 24 000). (d) Direct sequencing of KRT1 revealed that patient 1 (proband) and patient 2 (mother) were heterozygous for a single nucleotide substitution c.1434G→T (p.Glu478Asp) in exon 7.

On the basis of these clinicopathological findings in two affected members of the family, the diagnosis of IBS was strongly suspected, although the PPK findings were atypical of IBS. We performed direct sequencing of KRT2E. DNA was isolated by standard methods from the peripheral blood of patient 1 (proband) and patient 2 (mother), and was amplified by polymerase chain reaction (PCR). PCR products were subjected to ABI 310 automated sequencing (Perkin Elmer-ABI, Foster City, CA, U.S.A.). No mutation was found throughout the entire exons and exon/intron borders of KRT2E (Genbank accession no. NP000414) in either patient 1 or patient 2. We therefore sequenced KRT1 and KRT10 (Genbank accession nos. NT029419 and NT010755) and detected a heterozygous single nucleotide substitution (G to T) at base pair position 1434 in exon 7 of KRT1 (c.1434G→T) in both patients 1 and 2 (Fig. 2d). This exchange resulted in a substitution of a glutamic acid residue (GAG) to aspartic acid residue (GAT) at codon 478 (p.Glu478Asp), which was located 15 amino acids upstream of the end of the 2B rod domain in K1. No other pathogenic mutations were detected within the helix initiation and termination motifs of K1, K10 or the linker domain (L12) of K10 in the patients' DNA. This mutation was not found in 100 normal, unrelated alleles (from 50 Japanese individuals) by direct sequence analysis and was unlikely to be a polymorphism.

Interestingly, our patients showed features reminiscent of IBS both clinically and histopathologically. Their skin displayed granular degeneration restricted to the uppermost spinous and granular layers and superficial blistering resembling superficially denuded areas often observed in IBS.[7,8] In IBS, mutant K2e is expressed mainly in the uppermost spinous and granular layer keratinocytes, resulting in the formation of keratin clumps, granular degeneration and acantholysis in the superficial epidermis.[4–6] However, in the present family, a missense mutation p.Glu478Asp was detected in the 2B helix termination motif. An identical mutation was previously reported in a BCIE patient[9] who also showed superficial blisters and granular degeneration restricted to the uppermost spinous and granular layers[9] and the resulting phenotype appeared to be very similar in the two families with the identical mutation p.Glu478Asp. This fact is very important since it demonstrates a clear genotype/phenotype correlation.

Using *in vitro* keratin filament assembly experiments, Yang *et al.*[9] demonstrated that residual filament-forming ability was present in the p.Glu478Asp mutant peptide. The remaining ability is probably linked to the mild phenotype in the family, although the lysine substitution at the same glutamic acid residue (p.Glu478Lys) resulted in the typical BCIE phenotype.[10]

The present patients had PPK, although clear and obvious PPK has not been reported in IBS. Thus, PPK was a clinical feature that might indicate a diagnosis of BCIE in the present family.

We previously reported that the differential diagnosis of IBS and BCIE is difficult in some instances and molecular genetic testing is useful to differentiate IBS from BCIE.[7] In the present report, we show that p.Glu478Asp in K1 leads to clinicopathological features mimicking IBS, i.e. superficial blisters and granular degeneration restricted to the uppermost spinous and granular layers of the epidermis. These results further confirm that there are many cases of BCIE and IBS in which the correct differential diagnosis is problematic unless mutational analysis is performed.

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References

1 Smith F. The molecular genetics of keratin disorders. *Am J Clin Dermatol* 2003; **4**:347–64.
2 Lane EB, McLean WHI. Keratins and skin disorders. *J Pathol* 2004; **204**:355–66.
3 Ishida-Yamamoto A, McGrath JA, Judge MR *et al*. Selective involvement of keratins K1 and K10 in the cytoskeletal abnormality of epidermolytic hyperkeratosis (bullous congenital ichthyosiform erythroderma). *J Invest Dermatol* 1992; **99**:19–26.
4 Rothnagel JA, Traupe H, Wojcik S *et al*. Mutations in the rod domain of keratin 2e in patients with ichthyosis bullosa of Siemens. *Nat Genet* 1994; **7**:485–90.
5 Kremer H, Zeeuwen P, McLean WHI *et al*. Ichthyosis bullosa of Siemens is caused by mutations in the keratin 2e gene. *J Invest Dermatol* 1994; **103**:286–9.
6 McLean WHI, Morley SM, Lane EB *et al*. Ichthyosis bullosa of Siemens – a disease involving keratin 2e. *J Invest Dermatol* 1994; **103**:277–81.
7 Akiyama M, Tsuji-Abe Y, Yanagihara M *et al*. Ichthyosis bullosa of Siemens: its correct diagnosis facilitated by molecular genetic testing. *Br J Dermatol* 2005; **152**:1353–6.
8 Traupe H, Kolde G, Hamm H, Happle R. Ichthyosis bullosa of Siemens: a unique type of epidermolytic hyperkeratosis. *J Am Acad Dermatol* 1986; **14**:1000–5.
9 Yang JM, Nam K, Kim HC *et al*. A novel glutamic acid to aspartic acid mutation of the 2B rod domain in the keratin 1 chain in epidermolytic hyperkeratosis. *J Invest Dermatol* 1999; **112**:376–9.
10 Sun XK, Ma LL, Xie YQ *et al*. Keratin 1 and keratin 10 mutations causing epidermolytic hyperkeratosis in Chinese patient. *J Dermatol Sci* 2002; **29**:195–200.

Key words: *epidermolytic hyperkeratosis, granular degeneration, ichthyosis, palmoplantar keratoderma*

Conflicts of interest: none declared.

Primary cutaneous apocrine carcinoma presenting as carcinoma erysipeloides

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Sir, Primary cutaneous apocrine carcinoma of the skin can pose a diagnostic and therapeutic challenge. We report a case of a primary cutaneous apocrine carcinoma that masqueraded as cellulitis. A 90-year-old man with a history of colon adenocarcinoma presented with a left facial lesion which had rapidly spread to his anterior neck and the contralateral cheek over 6 weeks. Although the patient denied any constitutional symptoms, he experienced tightness over his lower face and neck. A well-demarcated, erythematous, firm plaque was present on the inferior margins of both cheeks and anterior neck (Fig. 1), with woody oedema overlying the involved areas. Breast examination did not reveal any abnormality.

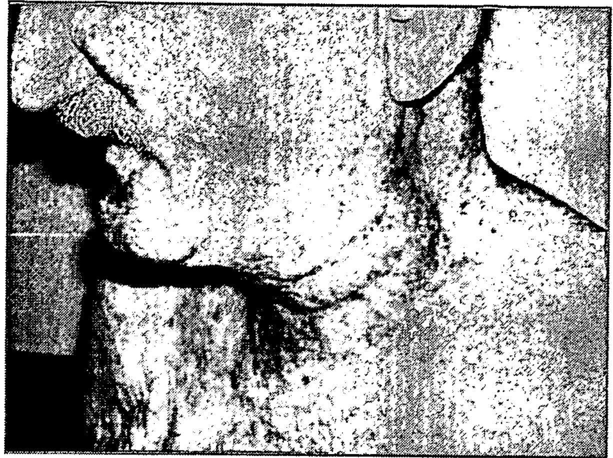

Fig 1. A well-demarcated, erythematous plaque with firm woody oedema involving the cheeks, chin and anterior neck.

Our initial clinical differential diagnosis included allergic contact dermatitis, erysipelas, cellulitis, panniculitis and carcinoma erysipeloides. Histology revealed single, and sheets of, pleomorphic cells with eosinophilic fine granules infiltrating the dermis and the subcutaneous tissue, sparing the epidermis (Fig. 2). On immunohistochemistry, these tumour cells stained positively for epithelial membrane antigen (EMA), pancytokeratin, cytokeratin isotype (CK) 7 and gross cystic disease fluid protein-15 (GCDFP-15), but negatively for oestrogen and progesterone receptors. The tumour lacked melanocytic (MART-1, S100) and lymphoid markers (CD45, CD43, CD30 and CD79a), carcinoembryonic antigen (CEA), CK5/6, CK20, E-cadherin, prostate-specific antigen, thyroid transcription factor-1 and mucicarmine. Human epidermal growth factor receptor-2 (HER2/neu) protein was moderately positive (2+). Subsequent fluorescence *in situ* hybridization study did not show amplification. Computed tomography scans revealed an ill-defined mass over the left cheek invading the buccinator muscles with a prominent left parotid lymph node. Bone scan did not show any evidence of metastasis. A thorough otolaryngological examination revealed no vocal cord involvement.

The patient was diagnosed with a poorly differentiated apocrine carcinoma. Despite trastuzumab infusions at 4 mg kg^{-1} over 4 weeks, the tumour enlarged. A 2-week trial of capecitabine 1250 mg m^{-2} twice daily was started. The tumour significantly regressed in size initially. He also noted softening of his skin, allowing greater range of movement upon mastication. Unfortunately, the patient had a relapse 1 month later.

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Epithelial and Mesenchymal Cell Biology

Expression of the Keratinocyte Lipid Transporter ABCA12 in Developing and Reconstituted Human Epidermis

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Serious defects in the epidermal keratinocyte lipid transporter ABCA12 are known to result in a deficient skin lipid barrier, leading to harlequin ichthyosis (HI). HI is the most severe inherited keratinizing disorder and is frequently fatal in the perinatal period. To clarify the role of ABCA12, ABCA12 expression was studied in developing human skin and HI lesions artificially reconstituted in immunodeficient mice. By immunofluorescent study, ABCA12 was expressed in the periderm of the early stage two-layered human fetal epidermis. After formation of a three-layered epidermis, ABCA12 staining was seen throughout the entire epidermis. ABCA12 mRNA expression significantly increased during human skin development and reached 62% of the expression in normal adult skin, whereas the expression rate of transglutaminase 1, loricrin, and kallikrein 7 remained low. We transplanted keratinocytes from patients with HI and succeeded in reconstituting HI skin lesions in immunodeficient mice. The reconstituted lesions showed similar changes to those of patients with HI. Our findings demonstrate that ABCA12 is highly expressed in fetal skin and suggest that ABCA12 may play an essential role under both the wet and dry conditions, including the dramatic turning point from a wet environment of the amniotic fluid to a dry environment after birth. ***(Am J Pathol 2007, 171:43–52; DOI: 10.2353/ajpath.2007.061207)***

One important event during terminal differentiation of stratified squamous epithelia such as the epidermis is the formation of intercellular lipid layers in the stratum corneum. Intercellular lipid layers in the stratum corneum are essential for epidermal barrier function. The lipid layers are formed from the extruded lipid contents secreted from lamellar granules within granular layer keratinocytes.

The ATP-binding cassette (ABC) transporter superfamily is one of the largest gene families, encoding a highly conserved group of proteins involved in energy-dependent (active) transport of a variety of substrates across biological membranes, including ions, amino acids, peptides, carbohydrates, and lipids.[1–3] ABC transporters have nucleotide binding folds located in the cytoplasm and use energy from ATP to transport substrates across the cell membrane.[4] ABC genes are widely dispersed throughout the eukaryotic genome and are highly conserved between species.[5,6] The ABCA subfamily comprises 12 full transporter proteins and one pseudogene (ABCA11). The ABCA subclass has received considerable attention, because mutations in these genes have been implicated in several human genetic diseases.[7–11] Recent studies have clarified that many members of the ABCA subclass play an important role in endogenous lipid transport.[12–17]

In 2005, ABCA12, a member of the ABCA subfamily, was reported to underlie harlequin ichthyosis (HI), one of the most devastating genodermatoses.[18,19] HI was known to show several morphological abnormalities reflecting defective lipid content: absent or abnormal lamellar granules in the granular keratinocytes, lipid droplets in the stratum corneum, and a lack of extracellular lipid lamellae.[20–26] We demonstrated that ABCA12 works as an epidermal keratinocyte lipid transporter and that defective ABCA12 results in a loss of the skin lipid barrier, leading to HI.[18]

In HI skin, epidermal morphogenesis already shows significant alterations *in utero*.[20,27–31] Affected neonates usually show the most severe, life-threatening symptoms such as large, thick, platelike scales over the whole body, ectropion, eclabium, and flattened ears from birth.[32] Pa-

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tients with HI usually die during the first few weeks of life from secondary infection, severe anemia, dehydration, circulatory disturbance, or renal failure. However, once patients with HI have survived beyond the perinatal period, their skin symptoms tend to be less severe, and some long-term survivors even show clinical features of the milder nonbullous congenital ichthyosiform erythroderma.[33,34] Some survivors can even stop taking oral retinoids. From these clinical findings, we hypothesized that ABCA12 deficiency is the most critical at around the time of birth and that the negative effects of ABCA12 deficiency might be reduced or compensated for after the baby grows beyond the perinatal period.

In the present study, to clarify further the pathomechanisms of severe HI manifestations from birth and the critical role of ABCA12 in the neonatal period, ABCA12 expression was studied in detail in developing human skin and artificially reconstituted HI lesions grown on immunodeficient mice. This is the first report describing ABCA12 expression during embryonic and fetal skin ontogeny, and it demonstrates that ABCA12 is highly expressed in the upper epidermis from the second trimester. Furthermore, we succeeded in establishing a model system for regenerated HI lesions harboring ABCA12 mutations in adult skin with reduced ABCA12 expression in the reconstituted HI skin lesions. The skin lesions reconstituted in the dry environment were similar to the original lesions seen at birth. The present results suggest that ABCA12 may play an essential role both in the wet conditions during fetal development and in the dry conditions including the dramatic turning point from wet condition in the amniotic fluid to dry environment around the birth.

Materials and Methods

Human Fetal Skin Specimens

Normal human fetal tissue was acquired (after informed consent was obtained) from Sapporo Maternity-Women's Hospital (Sapporo, Japan). Human embryonic and fetal skin specimens were obtained from abortuses of 7 to 22 weeks estimated gestational age (EGA). An HI fetal skin sample was obtained from an abortus at 21 weeks EGA that had been diagnosed with HI by prenatal skin biopsy.[30] Skin specimens were taken from the trunk, scalp, and fingers and processed for the present study. EGA was determined from maternal history, fetal measurements (crown, rump, and foot length), and comparative histological appearance of the epidermis.

Antibodies

Immunofluorescence labeling was performed as described below. We used anti-ABCA12 antisera[18] as a primary antibody. For control immunostaining, we also used mouse monoclonal anti-transglutaminase 1 (TGase1) antibody BT-621 (Biomedical Technologies, Inc., Stoughton, MA), because TGase1 is a major keratinization marker that is known to cross-link several precursor proteins in the formation of the cornified cell envelope during keratinocyte differentiation. Rabbit anti-human glucosylceramide antibody (Glycobiotech, Kükels, Germany) was used to clarify the expression sites of the lipid in the epidermis during development. Immunolabeling for keratin 10, a keratinization marker, and cathepsin D, a component of lamellar granules, was performed using mouse anti-human keratin 10 antibody (DAKO, Glostrup, Denmark) and rabbit anti-human cathepsin D antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in normal and HI fetal skin. Rat anti-human HLA class I antibody (Serotec Ltd., Oxford, UK) was also used to differentiate human keratinocyte-derived epidermis from host murine epidermis.

Immunofluorescent Labeling

Immunofluorescent labeling was performed as previously described.[35] In brief, 6-μm-thick sections of fresh skin samples cut using a cryostat were prepared for immunolabeling. Sections were fixed in 4% paraformaldehyde for 15 minutes at 4°C for labeling with anti-human HLA class I antibody or in acetone for 10 minutes at room temperature for labeling with other antibodies (ABCA12, TGase1, keratin 10, and cathepsin D) except for anti-human glucosylceramide antibody. We performed glucosylceramide labeling without any fixation. The sections were incubated in primary antibody solution for 2 hours at room temperature. Primary antibodies and dilutions were as follows: rabbit polyclonal anti-human ABCA12 antibody,[18] 1:800; mouse monoclonal anti-TGase1 antibody, BT-621, 1:100; rabbit polyclonal anti-human glucosylceramide antibody, 1:10; mouse polyclonal anti-human keratin 10 antibody, 1:100; rabbit polyclonal anti-human cathepsin D antibody, 1:10; and rat anti-human HLA class I antibody, 1:100. The sections were then incubated in each secondary antibody: fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin, anti-rabbit immunoglobulin, anti-rat immunoglobulin, or tetramethylrhodamine-5-(and -6)-isothiocyanate-conjugated donkey anti-rabbit immunoglobulin (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted 1:100 for 2 hours at room temperature, followed by 10 μg/ml TO-PRO-3 iodide (Molecular Probes, San Diego, CA) or propidium iodide (Dojindo Laboratories, Kumamoto, Japan) to counterstain nuclei for 10 minutes at 37°C. Sections were observed under an Olympus FluoView confocal laser scanning microscope (Olympus, Tokyo, Japan).

Real-Time Polymerase Chain Reaction Analysis

To quantify the ABCA12 mRNA expression levels together with TGase1, loricrin, and kallikrein 7 (KLK7) in fetal skin, total RNA was extracted from fresh skin samples. Commercial epidermal mRNA obtained from Stratagene (La Jolla, CA) was used only for the specimen at 18 and 20 weeks EGA. In addition, total RNA was extracted from fresh skin samples obtained from a human adult (a generally healthy Japanese male without any skin disease) at a surgical operation of a benign subcutaneous tumor, and the RNA sample was used for the real-

time polymerase chain reaction analysis. RNA samples were analyzed by the ABI prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Primers and probes specific for ABCA12, TGase1, loricrin, and KLK7 were obtained from the TaqMan gene expression assay (Applied Biosystems: Hs00292421_m1, Hs00165929_m1, Hs01894962_s1 and Hs00192503_m1).

Differences between the mean CT values of ABCA12, TGase1, loricrin, and KLK7 and those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, or large ribosomal protein (Applied Biosystems) were calculated as $\Delta CT_{sample} = CT_{ABCA12\ (or\ other\ keratinization\ markers)} - CT_{GAPDH\ (or\ other\ housekeeping\ genes)}$ and those of ΔCT for the normal adult skin as $\Delta CT_{calibrator} = CT_{ABCA12\ (or\ other\ keratinization\ markers)} - CT_{GAPDH\ (or\ other\ housekeeping\ genes)}$. Final results for fetal skin sample/adult skin (%) were determined by $2^{-(\Delta Ctsample\ -\ \Delta CTcalibrator)}$.

Skin Reconstruction from Normal Human Keratinocytes and HI Patients' Keratinocytes with Normal Fibroblasts

Normal human fibroblasts and keratinocytes were purchased from Kurabo (Osaka, Japan). We established primary cultures of skin cells from two patients with HI. One patient harbored a homozygous splice site mutation c.3295-2A>G and the other harbored heterozygous mutations: p.Ser387Asn and c.4158_4160del (p.Thr1387del) as previously reported.[36] In detail, patients' keratinocytes were isolated from lesional epidermis after separation from the dermis by overnight treatment of dispase I (Godoshusei, Chiba, Japan). After 0.25% trypsin digestion for 5 minutes, epidermal cells were collected and cultured in defined keratinocyte serum-free medium (Invitrogen, San Diego, CA). Normal human keratinocytes were grown in the same culture medium. Normal human fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics. All of the cells were maintained at 37°C in 5% CO_2 atmosphere.

Engraftment was performed as previously described.[37] Equal numbers of keratinocytes (normal human keratinocytes or keratinocytes from patients with HI) and normal human fibroblasts were combined at a final density of 6 to 8×10^6 cells, and the cells were thoroughly mixed. This cell slurry was engrafted into a silicon chamber attached to the back of an anesthetized severe combined immunodeficient mouse (Clea, Tokyo, Japan). After 1 week, the wounds had healed, and the chamber tops were removed. The skin reconstitution was completed 2 to 3 weeks thereafter.

We succeeded in reconstituting HI skin using keratinocytes from a patient with HI who had a homozygous mutation, c.3295-2A>G,[18] using the methods described above. Thus, with the same methods, we reconstituted HI lesions using keratinocytes from another patient with HI who had heterozygous mutations affecting both ABCA12 alleles, p.Ser387Asn and c.4158_4160del (p.Thr1387del) (see Ref. 37 for further detailed analysis of the reconstituted lesions).

Transmission Electron Microscopy

For transmission EM, fresh biopsies of fetal skin and reconstituted skin were fixed in 5% glutaraldehyde solution, postfixed in 1% OsO_4, dehydrated, and embedded in Epon 812. All of the samples were ultrathin-sectioned at a thickness of 70 nm and stained with uranyl acetate and lead citrate. Photographs were taken using a Hitachi H-7100 transmission electron microscope.

This study was approved by the medical ethical committees of Hokkaido University Graduate School of Medicine, Sapporo, Japan. The study was conducted according to the Declaration of Helsinki Principles.

Results

ABCA12 Expressed in the Periderm of Early Developing Epidermis and in the Upper Epidermis at the Later Stages

ABCA12 expression was seen in the periderm during the early period when the two-layered epidermis forms, about 6 to 9 weeks EGA (Figure 1B). In the two-layered epidermis, both ABCA12 and TGase1 were expressed only in periderm cells (Figure 1, A–D). In the three-layered epidermis (10 to 13 weeks EGA), ABCA12 staining was seen in the entire epidermis, including intense periderm staining, whereas TGase1 staining was restricted to the periderm (Figure 1, E–H). A similar pattern was observed in the period of four or more layered epidermis before keratinization (14 to 22 weeks EGA) (Figure 1, I–P). In the newborn skin, ABCA12 and TGase1 staining were restricted to upper layers of epidermis, mainly granular layers (Figure 1, Q–T). These staining patterns are similar to those in normal adult skin (Figure 1, U–X) as previously reported.[18]

Glucosylceramide Expression in Normal Fetal Skin and Its Reduction in HI Fetal Skin

We performed immunofluorescent staining of glucosylceramide, one of the most important precursors of ceramide, in normal and HI fetal skin. In normal fetal skin at 22 weeks EGA, the expression of glucosylceramide was observed in the upper epidermis, including periderm (Figure 2C). HI fetal skin at 21 weeks EGA shows marked hyperkeratosis by hematoxylin and eosin (H&E) staining (Figure 2B). In HI fetal skin, the expression of glucosylceramide was obviously reduced, and only weak expression was seen in the upper epidermis (Figure 2D), although the expression of keratin 10, a keratinization marker, and cathepsin D, a component of lamellar granules, in HI fetal skin (Figure 2, F and H) were similar to those in normal fetal skin (Figure 2, E and G). Thus, the reduced glucosylceramide expression was thought to be a specific change resulting from an abnormality in HI epidermis and was specifically caused by an ABCA12 deficiency in HI fetal skin.

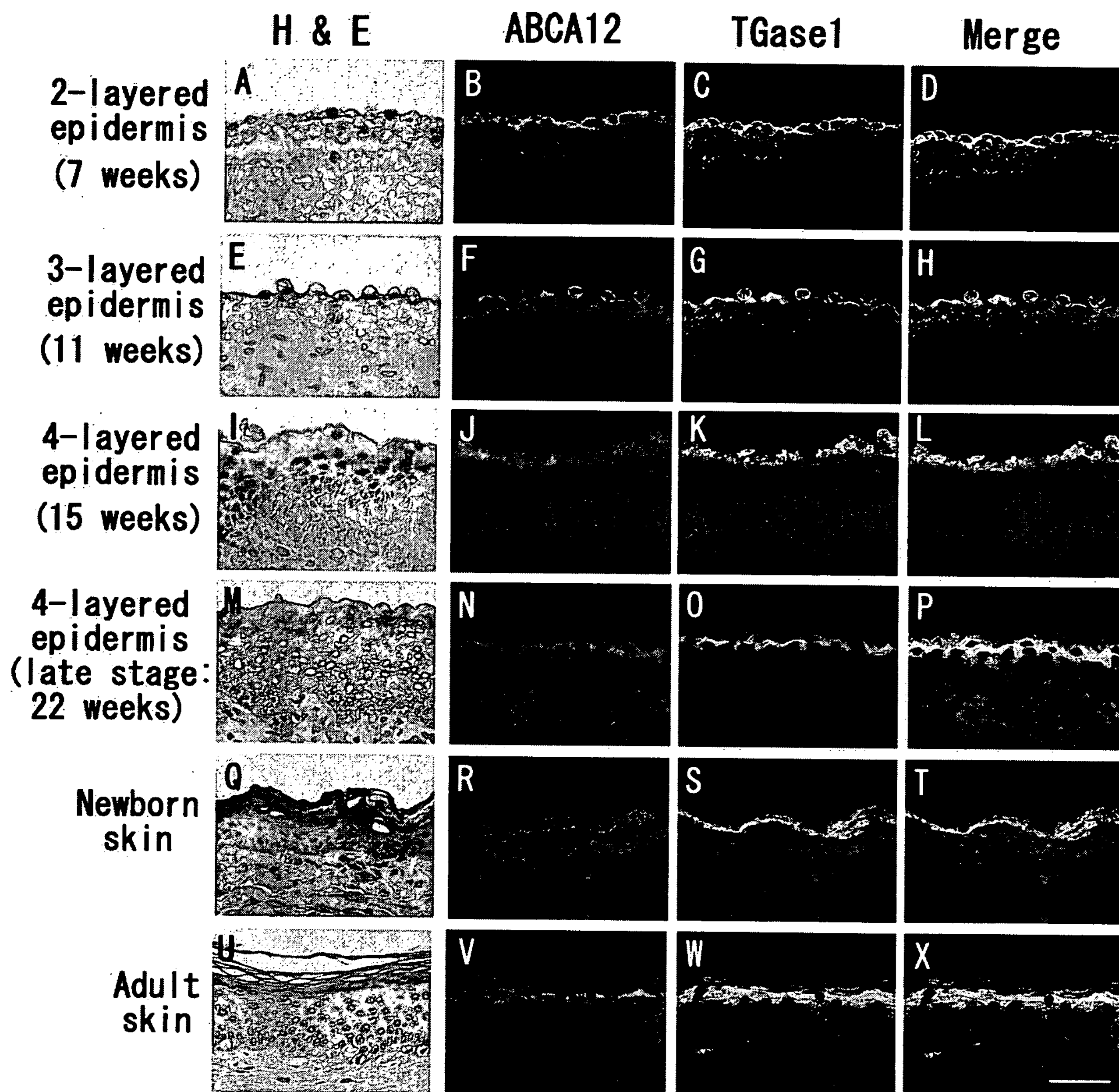


Figure 1. Expression of ABCA12 and TGase1 in developing skin. Fetal skin samples of 49 to 154 days EGA and newborn skin were double-stained for ABCA12 (red) and TGase1 (green). To show the anatomy of the sections clearly, photos of H&E-stained sections were included (**A**, **E**, **I**, **M**, **Q**, and **U**). **A–D:** In the two-layered epidermis (7 weeks EGA), both ABCA12 and TGase1 are expressed only in periderm cells. In the three-layered epidermis (11 weeks EGA; **E–H**), ABCA12 staining is seen in the entire epidermis, especially intensely within the periderm layer, whereas TGase1 staining remains only in the periderm. A similar pattern is observed in the four or more layered epidermis period (15 and 22 weeks EGA; **I–P**). **Q–T:** In the newborn skin, ABCA12 and TGase1 are seen only in the upper layer of the epidermis, mainly in the granular layers. These staining patterns are similar to those in normal adult skin (**U–X**). ABCA12, red (tetramethylrhodamine-5-(and -6)-isothiocyanate); TGase1, green (FITC); nuclear stain, blue (TO-PRO-3 iodide). Scale bar = 50 μm.

Many Vesicles Seen in the Periderm

In two-layered epidermis (6 to 9 weeks EGA) (Figure 3, A and B), the periderm contained many vesicles (arrows). In the three-layered epidermal stage (10 to 13 weeks EGA) (Figure 3, C and D), vesicles were observed at the cell periphery of periderm cells. In four or more layered epidermis before keratinization (14 to 22 weeks EGA) (Figure 3, E and F), the number of vesicles close to the cell membrane significantly increased. Due to the intense ABCA12 staining in the cytoplasm of periderm cells, some of the vesicles were thought to be associated with ABCA12 staining seen in this period, although a large number of these vesicles are thought to be pinocytic vesicles. The thickening of the periderm cell membrane was markedly observed in the four-layered epidermis (Figure 3F).

Increased ABA12 mRNA Expression in Fetal Skin at 15 Weeks EGA

We examined the expression of ABCA12, TGase1, loricrin, and KLK7 mRNA by real-time reverse transcriptase-polymerase chain reaction. The results were normalized

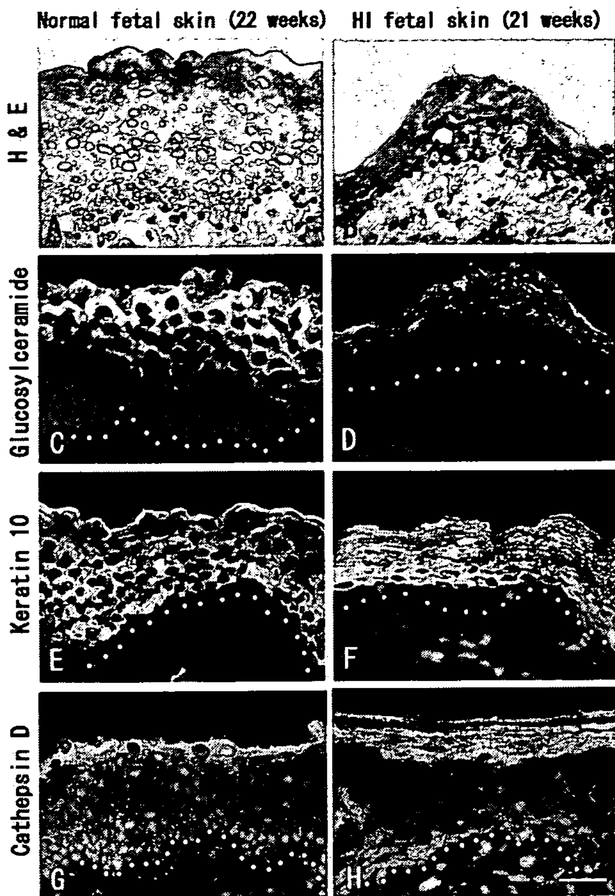


Figure 2. Glucosylceramide expression was confirmed in normal fetal epidermis at 22 weeks EGA, although the expression was remarkably weak in HI fetal epidermis. H&E staining (**A** and **B**) and immunofluorescent staining (**C–H**) in normal and HI fetal skin. In normal fetal skin at 22 weeks EGA, glucosylceramide was seen in the upper epidermis, including periderm (**C**). In contrast, the expression of glucosylceramide was significantly reduced in the upper epidermis of HI fetal skin at 21 weeks EGA (**D**). The expression of keratin 10 (**E** and **F**) and cathepsin D (**G** and **H**) showed no apparent difference in normal and HI fetal skin. Black and white dots indicate basement membrane. Glucosylceramide, keratin 10, and cathepsin D, green (FITC); nuclear stain, red (propidium iodide). Scale bar = 30 μm.

by expression of three housekeeping genes, GAPDH, β-actin, and large ribosomal protein, and the expression level of each mRNA was converted to a percentage rate compared with that of the normal adult skin. The expression level of ABCA12 mRNA normalized to GAPDH remarkably increased after 15 weeks EGA when compared with an earlier developmental stage (10 weeks EGA) (Figure 4). Expression rates of other keratinization-related molecules studied for controls, TGase1, loricrin, and KLK7, remained very low, whereas the expression rate of ABCA12 increased by up to 62% (at 18 and 20 weeks EGA) during development. The expression level of loricrin was very low (expression level at 18 and 20 weeks EGA/expression level in adult = 0.019%), probably due to the extremely high expression level of loricrin in adult samples. The results normalized to other housekeeping genes, β-actin, or large ribosomal protein, showed a similar pattern. ABCA12 mRNA expression during development became up-regulated to 86% (normalized to β-actin) or 62% (normalized to large ribosomal protein),

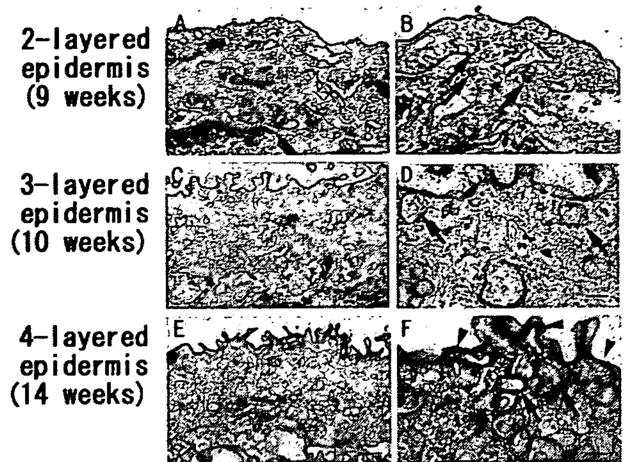


Figure 3. Electron microscopic findings of human developing epidermis. **A** and **B:** In the two-layered epidermis (9 weeks EGA), the periderm contains many vesicles (**arrows**). **C** and **D:** In the three-layered epidermis (10 weeks EGA), vesicles (**arrows**) are observed at the periderm cell periphery. **E** and **F:** In the four-layered epidermis (14 weeks EGA), the number of vesicles (**arrows**) close to the cell membrane significantly increased. The thickening of the periderm cell membrane (**arrowheads**) was seen in the four-layered epidermis (**F**). Scale bars: 400 nm (**A**, **C**, and **E**) and 200 nm (**B**, **D**, and **F**).

whereas TGase1, loricrin, and KLK7 remained very low (data not shown). This increase in ABCA12 mRNA expression is consistent with ABCA12 immunofluorescence findings during human epidermal development.

Remarkable Hyperkeratosis in Reconstituted HI Lesions

We regenerated normal human skin using cultured normal human keratinocytes and fibroblasts. Four weeks after transplantation, the grafts exhibited an ordinary skin

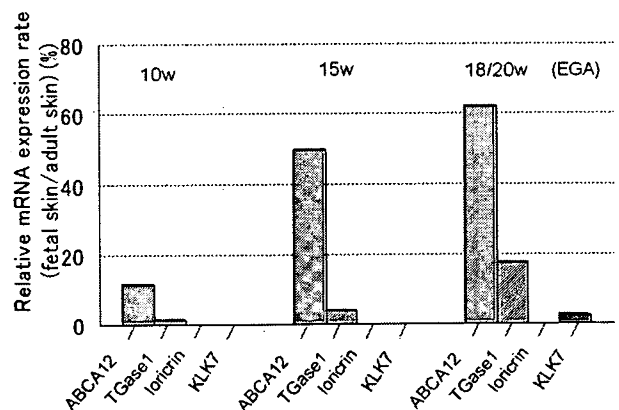
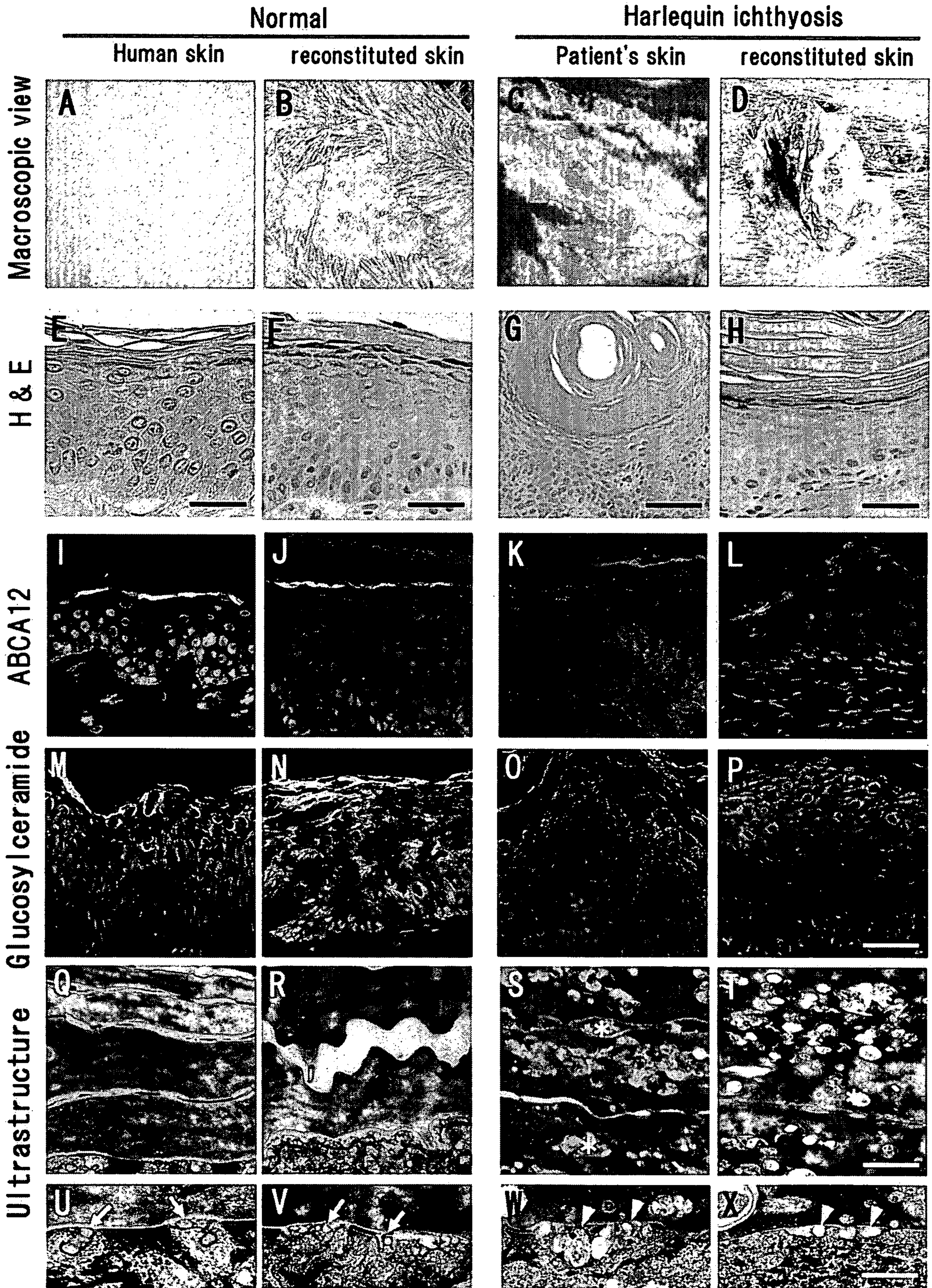


Figure 4. Expression of ABCA12 was higher in fetal skin compared with those of other keratinization markers. The mRNA expression of ABCA12, TGase1, loricrin, and KLK7 in fetal skin was studied by real-time polymerase chain reaction analysis, normalized by GAPDH. mRNA expression rates in fetal skin compared with adult skin expression level (fetal skin/adult skin) were summarized in the graph. At 15 weeks EGA, the rate of ABCA12 mRNA expression was significantly increased compared with that at 10 weeks EGA, which reached to almost 62% at 18 and 20 weeks EGA. On the other hand, mRNA expression rates of TGase1, loricrin, and KLK7 remained low during fetal skin development. Each expression rate at 18 and 20 weeks EGA was as follows: TGase1, 17%; loricrin, 0.019%; KLK7, 2.4%. This result was consistent with that using two other housekeeping genes, β-actin, and large ribosomal protein.


Normal
Harlequin ichthyosis
Human skin
reconstituted skin
Patient's skin
reconstituted skin
Macroscopic view
H & E
ABCA12
Glucosylceramide
Ultrastructure
A
B
C
D
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F
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appearance (Figure 5B). Immunohistochemical staining for anti-human HLA class I showed that the reconstituted epidermis and dermis had been organized by surviving human cells (data not shown). An epidermis with a morphology resembling that of normal epidermis was observed in reconstituted skin using normal control keratinocytes and normal human fibroblasts (Figure 5, E and F). Frozen sections stained with H&E showed that the reconstituted epidermis composed of keratinocytes with normal morphology was of a normal thickness (Figure 5F), although the dermal band of regenerated human cells was thin.

We regenerated HI skin lesions using cultured keratinocytes harboring ABCA12 mutations p.Ser387Asn and c.4158_4160del (p.Thr1387del) and normal human fibroblasts. Four weeks after transplantation, the grafts exhibited a rugged external epidermal surface and marked hyperkeratosis (Figure 5D). Immunohistochemical staining for anti-human HLA class I showed that the reconstituted epidermis and dermis were organized by human cells (data not shown). Frozen sections with H&E staining showed that the morphology of the reconstituted skin composed of cells from a patient with HI revealed a thickened stratum corneum (Figure 5H), similar to that in the original HI patient lesions (Figure 5G). From the basal layers to the granular layers, there were no differences between reconstituted skin from HI patient keratinocytes and normal human keratinocytes.

Reduced ABCA12 Expression in Reconstituted HI Skin

Strong ABCA12 expression was observed in the granular layers of the normal human skin (Figure 5I), and a similar pattern was seen in reconstituted normal human skin (Figure 5J). Significantly reduced ABCA12 expression was observed in the granular layers of the skin of the patient with HI (Figure 5K). In the reconstituted HI lesional epidermis, only weak ABCA12 expression (Figure 5L) was seen, similar to that in the skin of the patient with HI. Very weak ABCA12 immunostaining was seen in the spinous layers in all samples (Figure 5, I–L). Spinous layer ABCA12 staining intensity showed no differences between control or the HI samples.

Weak Glucosylceramide Expression in HI Patien's Skin and in Reconstituted HI Skin

In normal human skin, strong glucosylceramide expression was seen in the upper epidermis, mainly in the granular layer (Figure 5M). Glucosylceramide was distributed broadly within the entire cytoplasm of the upper epidermal cells. A similar pattern was seen in the normal keratinocyte-reconstituted skin (Figure 5N).

In the skin of the patient with HI, only weak glucosylceramide expression was observed around nuclei in the upper epidermal cells (Figure 5O). A similar pattern of glucosylceramide expression was seen in the reconstituted HI skin lesion (Figure 5P).

Abnormal Lamellar Granules and Lipid Accumulation within the Resonstituted HI Epidermis

Ultrastructurally, in the normal reconstituted epidermis, keratin-filaggrin material occupied the cytoplasm of cornified cells, and no lipid vacuoles were seen in the cornified cell layers (Figure 5R). At the boundary between the granular and cornified cell layers, uniformly small lamellar granules containing lamellar structures were observed, and lamellar granule contents were secreted into the extracellular space (Figure 5V, arrows). In the cytoplasm of the granular layer cells, normal lamellar granules were seen (data not shown). All of these features were seen in the normal human epidermis *in vivo* (Figure 5, Q and U).

Similar to the skin of the patient with HI (Figure 5, S and W), the reconstituted epidermis using keratinocytes from the patient with HI always demonstrated multiple, typical features of HI skin, including abnormal lipid inclusions that were frequently observed in the cornified layer cells (Figure 5T, asterisks) and abnormal lamellar granules characteristic of HI that were also localized close to the extracellular space (Figure 5X, arrowheads). Some lipid inclusions in the cornified layer cells were apparently empty, although others contained electron-dense vesicular or granular material (Figure 5, S and T). In the upper spinous and granular layers, abnormal lamellar granules

Figure 5. Establishment of a reconstituted skin model for HI. **A–D:** Macroscopic features of human skin and regenerated skin. We regenerated human skin using cultured normal human keratinocytes (**B**) and cells from a patient with HI (**D**) on the back of severe combined immunodeficient mice. **E–H:** H&E staining of human skin and regenerated skin. Similar morphological features could be seen in the normal human skin (**E**) and reconstituted normal skin (**F**). The reconstituted HI skin grown from back lesions showed thick scales (**D**), although the hyperkeratosis was milder in the reconstituted HI grafted area (**H**) than the original HI patient skin (**G**). **I–L:** ABCA12 immunostaining. Strong ABCA12 expression (green) was observed in the granular layers in normal human skin (**I**) and reconstituted normal human skin (**J**). Significantly reduced ABCA12 expression was observed in the granular layers of the HI patient skin (**K**) and in the reconstituted HI lesion (**L**). Very weak ABCA12 immunostaining was seen in the cytoplasm of spinous layer cells in each section (**I–L**). ABCA12, green (FITC); nuclear stain, red (propidium iodide). **M–P:** Glucosylceramide immunostaining. Cytoplasmic staining for glucosylceramide (green) was seen in the upper epidermis of normal human skin (**M**) and of reconstituted normal skin (**N**). Only weak cytoplasmic staining for glucosylceramide was observed in the upper epidermis of HI patient's skin (**O**) and in the reconstituted HI lesion (**P**). Glucosylceramide, green (FITC); nuclear stain, red (propidium iodide). **Q–X:** Electron microscopic features of the cornified layers of the skin. **Q–T:** Ultrastructure of cornified cell layers. Accumulation of lipid vacuoles (**asterisks**) was observed in the cornified layer cells in the patient skin (**S**) and in the reconstituted HI lesion (**T**). Those vacuoles were not seen in normal skin (**Q**) or reconstituted normal skin (**R**). **U–X:** Ultrastructure of lamellar granules at the boundary between granular and cornified cell layers. Normal lamellar granules secreting their content to the extracellular space (**arrows**) were observed in the normal human skin (**U**) and the reconstituted normal skin (**V**). Abnormal lamellar granules (**arrowheads**) were seen in the granular layer cells both in the patient's skin (**W**) and in the reconstituted HI lesion (**X**). **A**, **E**, **I**, **M**, **Q**, and **U:** Normal human skin. **B**, **F**, **J**, **N**, **R**, and **V:** Skin regenerated by normal keratinocytes at 4 weeks after transplantation. **C**, **G**, **K**, **O**, **S**, and **W:** Original skin lesion from the HI patient whose keratinocytes were used for the reconstitution. **D**, **H**, **L**, **P**, **T**, and **X:** Skin regenerated from HI patient keratinocytes at 4 weeks after transplantation. Scale bars: 30 μm (**E**, **F**, **H**, and **P**), 100 μm (**G**), 500 nm (**T**), and 250 nm (**X**).

in the skin of patient with HI and reconstituted HI skin lacked the normal lamellar structure but contained electron-dense vesicular, granular, or irregularly shaped material (Figure 5, W and X). In normal human skin and normal reconstituted skin, intact lamellar granules with lamellar structures were observed fused with the cell membrane, and the contents of the lamellar granules were secreted into the intercellular space (Figure 5, U and V). Conversely, some abnormal lamellar granules in the skin of the patient with HI and reconstituted skin were observed as empty. In addition, HI keratinocytes and derived tissue showed abnormal vesicular lamellar granules that had become congested in the cytoplasm of granular layer cells (Figure 5, W and X).

Discussion

From the present results, we have demonstrated that ABCA12 is already expressed from the early stages of fetal epidermal development. We also found that ABCA12 staining showed an extended distribution covering the entire epidermis from the three-layered epidermal stage. The results were consistent with a previous report of another lamellar granule-associated protein, the antigen recognized by the AE17 antibody, which was also detected from the two-layered epidermal stage in the periderm[35] and in the underlying intermediate cell layers of the epidermis from the stage of three-layered epidermis. The sequential expression patterns of keratinization-related molecules in the periderm were associated with vesicles and the thickening of the cell membrane of periderm cells revealed by electron microscopy.[35] Moreover, the increased level of ABCA12 mRNA expression at 15 weeks EGA also reflected the ABCA12 immunofluorescent staining throughout the entire epidermis during human epidermal development. This increasing level and extended distribution of ABCA12 expression is unique compared with the expression of other keratinization-related proteins, such as TGase1, loricrin, and KLK7, which were not up-regulated during fetal skin development. In addition, post-110-day fetal epidermis was reported to be rich in ceramides, glycosphingolipids, triglycerides, and sterol esters.[38] By immunofluorescence study, glucosylceramide was expressed in the upper epidermis of the normal fetal skin, although the HI fetal skin at 21 weeks EGA showed marked reduction of glucosylceramide expression.

From these findings, we hypothesized that the periderm cells secrete lipid during their regression, similar to the granular layer cells of the adult epidermis. This putative role of the periderm is closely associated with an increase of ABCA12 expression before keratinization during human fetal skin development. We thought that ABCA12 and the related lipid transport system might play an important role under hydrated conditions in the amniotic fluid during development. However, we do not have enough data to support this hypothesis and, in previous reports, there was no apparent morphology abnormality in the periderm of HI fetuses.[20,26]

Furthermore, to confirm a role of ABCA12 in dry conditions after delivery, we reconstituted HI lesional skin from cultured patient keratinocytes harboring mutations in *ABCA12* under dry conditions, ie, on the back of immunodeficient mice using a silicone chamber. The reconstituted skin showed similar morphological features to HI patient skin lesions even in dry conditions. These grafts exhibited abnormal surface features, ie, rugged surface and marked hyperkeratosis typically sharing many features seen in the surface of HI patient skin. Histological analysis showed that the reconstituted skin composed of patient keratinocytes revealed an extraordinarily thick stratum corneum. Immunofluorescence studies of ABCA12 and glucosylceramide expression in reconstituted skin showed similar staining patterns to those of the original normal skin or HI patient skin. The abnormal distribution of glucosylceramide in reconstituted HI skin suggests that dysfunction of ABCA12 affects glucosylceramide transport, even in the reconstituted HI skin. Ultrastructurally, the reconstituted epidermis from the HI patient's cells showed abnormal granular layer lamellar granules and lipid droplets in the cornified layer cells. ABCA12 expression was remarkably reduced in the reconstituted skin similar to that seen in the HI patient skin lesion. Thus, we have generated and characterized a model system for HI skin lesions *in vivo* by regeneration of HI lesions using primary cultured patient keratinocytes.

From these findings, we have demonstrated that defective ABCA12 causes HI lesions even in dry conditions. Moreover, this system provides a powerful tool to analyze ABCA12 gene function and to evaluate various treatments for HI. This will also prove useful to develop more effective gene therapy approaches for HI.

Long-term survivors of HI usually show lamellar ichthyosis or an nonbullous congenital ichthyosiform erythroderma phenotype, which is milder than the typical HI phenotype, as patients become older. The precise mechanisms for this improvement are not well understood. However, we might expect a compensatory mechanism for ABCA12 deficiency, which might work in a dry environment after delivery but not under wet conditions, for example during fetal development. We expected that ABCA12 gene expression would be up-regulated in the dry environment and that residual activities of ABCA12 peptides from the mutant allele contribute to the improvement of the clinical features. However, in our present study, we failed to observe any apparent up-regulation of ABCA12 expression in the reconstituted HI skin model by immunofluorescent staining, compared with the original patient skin lesion. In this context, we predict that specific mechanisms other than a direct up-regulation of ABCA12 expression may compensate in the reconstituted skin in the dry environment.

In a separate disease entity, the self-healing collodion baby with tranglutaminase1 mutations, after birth, water molecules are naturally lost from the skin and the mutated TGase1 enzyme is predicted to isomerize back to a partially active *cis* form, resulting in recovery of the phenotype as a patient becomes older.[39] Of course, the compensatory mechanism for ABCA12 defects is likely to be different from TGase1 compensation in self-healing la-

mellar ichthyosis. As yet unknown lipid transporters and transport mechanisms other than ABCA12 may be involved in lipid transport, accumulation, and secretion in human keratinocytes. Further comprehensive studies are needed to clarify the alternative lipid transport mechanisms involved in the keratinization processes and to elucidate the compensatory mechanisms for ABCA12 deficiency in patients with HI.

Acknowledgments

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References

1. Allikmets R, Gerrard B, Hutchinson A, Dean M: Characterization of the human ABC superfamily: isolation and mapping of 21 new genes using the expressed sequence tags database. Hum Mol Genet 1996, 5:1649–1655
2. Borst P, Elferink RO: Mammalian ABC transporters in health and disease. Annu Rev Biochem 2002, 71:537–592
3. Dean M, Rzhetsky A, Allikmets R: The human ATP-binding cassette (ABC) transporter superfamily. Genome Res 2001, 11:1156–1166
4. Klein I, Sarkadi B, Varadi A: An inventory of the human ABC proteins. Biochim Biophys Acta 1999, 1461:237–262
5. Higgins CF: ABC transporters: from microorganisms to man. Annu Rev Cell Biol 1992, 8:67–113
6. Peelman F, Labeur C, Vanloo B, Roosbeek S, Devaud C, Duverger N, Denefle P, Rosir M, Van dekerckhove J, Rosseneu M: Characterization of the ABCA transporter subfamily: identification of prokaryotic and eukaryotic members, phylogeny and topology. J Mol Biol 2003, 325:259–274
7. Allikmets R, Singh N, Sun H, Shroyer NF, Hutchinson A, Chidambaram A, Gerrard B, Baird L, Stauffer D, Peiffer A, Rattner A, Smallwood P, Li Y, Anderson KL, Lewis RA, Nathans J, Leppert M, Dean M, Lupski JR: A photoreceptor cell-specific ATP-binding transporter gene (ABCR) is mutated in recessive Stargardt macular dystrophy. Nat Genet 1997, 15:236–246
8. Allikmets R, Shroyer NF, Singh N, Seddon JM, Lewis RA, Bernstein PS, Peiffer A, Zabriskie NA, Li Y, Hutchinson A, Dean M, Lupski JR, Leppert M: Mutation of the Stargardt disease gene (ABCR) in age-related macular degeneration. Science 1997, 277:1805–1807
9. Brooks-Wilson A, Marcil M, Clee SM, Zhang LH, Roomp K, van Dam M, Yu L, Brewer C, Collins JA, Molhuizen HO, Loubser O, Ouelette BF, Fichter K, Ashbourne-Excoffon KJ, Sensen CW, Scherer S, Mott S, Denis M, Martindale D, Frohlich J, Morgan K, Koop B, Pimstone S, Kastelein JJ, Genest Jr J, Hayden MR: Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency. Nat Genet 1999, 22:336–345
10. Rust S, Rosier M, Funke H, Real J, Amoura Z, Piette JC, Deleuze JF, Brewer HB, Duverger N, Denefle P, Assmann G: Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1. Nat Genet 1999, 22:352–355
11. Shulenin S, Nogee LM, Annilo T, Wert SE, Whitsett JA, Dean M: ABCA3 gene mutations in newborns with fatal surfactant deficiency. N Engl J Med 2004, 350:1296–1303
12. Orsó E, Broccardo C, Kaminski WE, Bottcher A, Liebisch G, Drobnik W, Gotz A, Chambenoit O, Diederich W, Langmann T, Spruss T, Luciani MF, Rothe G, Lackner KJ, Chimini G, Schmitz G: Transport of lipids from Golgi to plasma membrane is defective in tangier disease patients and Abc1-deficient mice. Nat Genet 2000, 24:192–196
13. Schmitz G, Langmann T: Structure, function and regulation of the ABC1 gene product. Curr Opin Lipidol 2001, 12:129–140
14. Kaminski WE, Piehler A, Pullmann K, Porsch-Ozcurumez M, Duong C, Bared GM, Buchler C, Schmitz G: Complete coding sequence, promoter region, and genomic structure of the human ABCA2 gene and evidence for sterol-dependent regulation in macrophages. Biochem Biophys Res Commun 2001, 281:249–258
15. Yamano G, Funahashi H, Kawanami O, Zhao LX, Ban N, Uchida Y, Morohoshi T, Ogawa J, Shioda S, Inagaki: ABCA3 is a lamellar body membrane protein in human lung alveolar type II cells. FEBS Lett 2001, 508: 221–225
16. Nagata K, Yamamoto A, Ban N, Tanaka AR, Matsuo M, Kioka N, Inagaki N, Ueda K: Human ABCA3, a product of a responsible gene for abca3 for fetal surfactant deficiency in new borns, exhibits unique ATP hydrolysis activity and generates intracellular multilamellar vesicles. Biochem Biophys Res Commun 2004, 324:262–268
17. Allikmets R: Simple and complex ABCR: genetic predisposition to retinal disease. Am J Hum Genet 2000, 67:793–799
18. Akiyama M, Sugiyama-Nakagiri Y, Sakai K, MacMillan JR, Goto M, Arita K, Tsuji-Abe Y, Tabata N, Matsuoka K, Sasaki R, Sawamura D, Shimizu H: Mutations in lipid transporter ABCA12 in harlequin ichthyosis and functional recovery by corrective gene transfer. J Clin Invest 2005, 115:1777–1784
19. Kelsell DP, Norgett EE, Unsworth H, Teh MT, Cullup T, Mein CA, Dopping-Hepenstal PJ, Dale BA, Tadini G, Fleckman P, Stephens KG, Sybert VP, Mallory SB, North BV, Witt DR, Sprecher E, Taylor AE, Ilchyshyn A, Kennedy CT, Goodyear H, Moss C, Paige D, Harper JI, Young BD, Leigh IM, Eady RA, O'Toole EA: Mutations in ABCA12 underlie the severe congenital skin disease harlequin ichthyosis. Am J Hum Genet 2005, 76:794–803
20. Akiyama M, Kim D-K, Main DM, Otto CE, Holbrook KA: Characteristic morphologic abnormality of harlequin ichthyosis detected in amniotic fluid cells. J Invest Dermatol 1994, 102:210–213
21. Dale BA, Holbrook KA, Fleckman P, Kimball JR, Brumbaugh S, Sybert VP: Heterogeneity in harlequin ichthyosis, an inborn error of epidermal keratinization: variable morphology and structural protein expression and a defect in lamellar granules. J Invest Dermatol 1990, 94:6–18
22. Milner ME, O'Guin WM, Holbrook KA, Dale BA: Abnormal lamellar granules in harlequin ichthyosis. J Invest Dermatol 1992, 99:824–829
23. Hashimoto K, Khan S: Harlequin fetus with abnormal lamellar granules and giant mitochondria. J Cutan Pathol 1992, 19:247–252
24. Hashimoto K, De Dobbeleer G, Kanzaki T: Electron microscopic studies of harlequin fetuses. Pediatr Dermatol 1993, 10:214–223
25. Akiyama M, Yoneda K, Kim S-Y, Koyama H, Shimizu H: Cornified cell envelop proteins and keratins are normally distributed in harlequin ichthyosis. J Cutan Pathol 1996, 23:571–575
26. Akiyama M, Dale BA, Smith LT, Shimizu H, Holbrook KA: Regional difference in expression of characteristic abnormality of harlequin ichthyosis in affected fetuses. Prenat Diagn 1998, 18:425–436
27. Shimizu A, Akiyama M, Ishiko A, Yoshiike T, Suzumori K, Shimizu H: Prenatal exclusion of harlequin ichthyosis; potential pitfalls in the timing of the fetal skin biopsy. Br J Dermatol 2005, 153:811–814
28. Blanchet-Bardon C, Dumez Y, Labbe F, Lutzner MA, Puissant A, Henrion R, Bernheim A: Prenatal diagnosis of harlequin ichthyosis. Lancet 1983, 1:132
29. Holbrook KA, Smith LT, Elias S: Prenatal diagnosis of genetic skin disease using fetal skin biopsy samples. Arch Dermatol 1993, 129:1437–1454
30. Akiyama M, Suzumori K, Shimizu H: Prenatal diagnosis of harlequin ichthyosis by the examinations of keratinized hair canals and amniotic fluid cells at 19 weeks' estimated gestational age. Prenat Diagn 1999, 19:167–171
31. Akiyama M, Holbrook A: Analysis of skin-derived amniotic fluid cells in the second trimester; detection of severe genodermatoses expressed in the fetal period. J Invest Dermatol 1994, 103:674–677
32. Williams ML, Elias PM: Genetically transmitted, generalized disorders of cornification; the ichthyoses. Dermatol Clin 1987, 5:155–178
33. Franco RC: Successful treatment of harlequin ichthyosis with acitretin. Int J Dermatol 2001, 40:472–484
34. Haftek M, Cambazard F, Dhouailly D, Reano A, Simon M, Lachaux A, Serre G, Claudy A, Schmitt D: A longitudinal study of a harlequin infant presenting clinically as non-bullous congenital ichthyosiform erythroderma. Br J Dermatol 1996, 135:448–453
35. Akiyama M, Smith LT, Yoneda K, Holbrook KA, Hohl D, Shimizu H: Periderm cells form cornified cell envelope in their regression pro-

cess during human epidermal development. J Invest Dermatol 1999, 112:903–909
36. Akiyama M, Sakai K, Sugiyama-Nakagiri Y, Yamanaka Y, McMillan JR, Sawamura D, Niizeki H, Miyagawa S, Shimizu H: Compound heterozygous mutations including a de novo missense mutation in ABCA12 led to a case of harlequin ichthyosis with moderate clinical severity. J Invest Dermatol 2006, 126:1518–1523
37. Sugiyama-Nakagiri Y, Akiyama M, Shimizu H: Hair follicle stem cell-targeted gene transfer and reconstitution system. Gene Ther 2006, 13:732–737
38. Williams ML, Hincenbergs M, Holdbrook KA: Skin lipid content during early fetal development. J Invest Dermatol 1988, 91:263–268
39. Raghunath M, Hennies HC, Ahvazi B, Vogel M, Reis A, Steinert PM, Traupe H: Self-healing collodion baby: a dynamic phenotype explained by a particular transglutaminase-1 mutation. J Invest Dermatol 2003, 120:224–228

Capon F, Semprini S, Chimenti S, Fabrizi G, Zambruno G, Murgia S *et al.* (2001) Fine mapping of the PSORS4 psoriasis susceptibility region on chromosome 1q21. *J Invest Dermatol* 116:728–30

Capon F, Semprini S, Dallapiccola B, Novelli G (1999) Evidence for interaction between psoriasis-susceptibility loci on chromosomes 6p21 and 1q21. *Am J Hum Genet* 65:1798–800

Capon F, Trembath RC, Barker JN (2004) An update on the genetics of psoriasis. *Dermatol Clin* 22:339–47, vii

Consortium IPG (2003) The International Psoriasis Genetics Study: assessing linkage to 14 candidate susceptibility loci in a cohort of 942 affected sib pairs. *Am J Hum Genet* 73:430–7

Cookson WO, Ubhi B, Lawrence R, Abecasis GR, Walley AJ, Cox HE *et al.* (2001) Genetic linkage of childhood atopic dermatitis to psoriasis susceptibility loci. *Nat Genet* 27:372–3

Elder JT, Nair RP, Henseler T, Jenisch S, Stuart P, Chia N *et al.* (2001) The genetics of psoriasis 2001: the odyssey continues. *Arch Dermatol* 137:1447–54

Fischer M, William T, Helmbold P, Wohlrab J, Marsch W (2004) Expression of epidermal *N*-methyl-D-aspartate receptors (NMDAR1) depends on formation of the granular layer – analysis in diseases with parakeratotic cornification. *Arch Dermatol Res* 296:157–62

Giardina E, Sinibaldi C, Chini L, Moschese V, Marulli G, Provini A *et al.* (2006) Co-localization of susceptibility loci for psoriasis (PSORS4) and atopic dermatitis (ATOD2) on human chromosome 1q21. *Hum Hered* 61:229–36

Henseler T, Christophers E (1995) Disease concomitance in psoriasis. *J Am Acad Dermatol* 32:982–6

Irvine AD, McLean WH (2006) Breaking the (un)sound barrier: filaggrin is a major gene for atopic dermatitis. *J Invest Dermatol* 126:1200–2

Jenisch S, Hampe J, Elder JT, Nair R, Stuart P, Voorhees JJ *et al.* (2006) CARD15 mutations in patients with plaque-type psoriasis and psoriatic arthritis: lack of association. *Arch Dermatol Res* 297:409–11

Jenisch S, Henseler T, Nair RP, Guo SW, Westphal E, Stuart P *et al.* (1998) Linkage analysis of human leukocyte antigen (HLA) markers in familial psoriasis: strong disequilibrium effects provide evidence for a major determinant in the HLA-B/-C region. *Am J Hum Genet* 63:191–9

Krawczak M, Nikolaus S, von Eberstein H, Croucher PJ, El Mokhtari NE, Schreiber S (2006) PopGen: population-based recruitment of patients and controls for the analysis of complex genotype–phenotype relationships. *Community Genet* 9:55–61

Marenholz I, Nickel R, Ruschendorf F, Schulz F, Esparza-Gordillo J, Kerscher T *et al.* (2006) Filaggrin loss-of-function mutations predispose to phenotypes involved in the atopic march. *J Allergy Clin Immunol* 118: 866–71

Nair RP, Stuart PE, Nistor I, Hiremagalore R, Chia NV, Jenisch S *et al.* (2006) Sequence and haplotype analysis supports HLA-C as the psoriasis susceptibility 1 gene. *Am J Hum Genet* 78:827–51

Palmer CN, Irvine AD, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee SP *et al.* (2006) Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat Genet* 38:441–6

Ruether A, Stoll M, Schwarz T, Schreiber S, Folster-Holst R (2006) Filaggrin loss-of-function variant contributes to atopic dermatitis risk in the population of Northern Germany. *Br J Dermatol* 155:1093–4

Sandilands A, O'Regan GM, Liao H, Zhao Y, Terron-Kwiatkowski A, Watson RM *et al.* (2006) Prevalent and rare mutations in the gene encoding filaggrin cause ichthyosis vulgaris and predispose individuals to atopic dermatitis. *J Invest Dermatol* 126:1770–5

Smith FJ, Irvine AD, Terron-Kwiatkowski A, Sandilands A, Campbell LE, Zhao Y *et al.* (2006) Loss-of-function mutations in the gene encoding filaggrin cause ichthyosis vulgaris. *Nat Genet* 38:337–42

Stemmler S, Parwez Q, Petrasch-Parwez E, Epplen JT, Hoffjan S (2007) Two common loss-of-function mutations within the filaggrin gene predispose for early onset of atopic dermatitis. *J Invest Dermatol* 127: 722–4

Trembath RC, Clough RL, Rosbotham JL, Jones AB, Camp RD, Frodsham A *et al.* (1997a) Identification of a major susceptibility locus on chromosome 6p and evidence for further disease loci revealed by a two stage genome-wide search in psoriasis. *Hum Mol Genet* 6:813–20

Trembath RC, Clough RL, Rosbotham JL, Jones AB, Camp RDR, Frodsham A *et al.* (1997b) Identification of a major susceptibility locus on chromosome 6p and evidence for further disease loci revealed by a two stage genome-wide search in psoriasis. *Hum Mol Genet* 6:813–20

van der Vleuten CJ, de Jong EM, van de Kerkhof PC (1996) Epidermal differentiation characteristics of the psoriatic plaque during treatment with calcipotriol. *Arch Dermatol Res* 288:366–72

Veal CD, Clough RL, Barber RC, Mason S, Tillman D, Ferry B *et al.* (2001) Identification of a novel psoriasis susceptibility locus at 1p and evidence of epistasis between PSORS1 and candidate loci. *J Med Genet* 38:7–13

Weidinger S, Illig T, Baurecht H, Irvine AD, Rodriguez E, Diaz-Lacava A *et al.* (2006) Loss-of-function variations within the filaggrin gene predispose for atopic dermatitis with allergic sensitizations. *J Allergy Clin Immunol* 118:214–9

Zhang XJ, Zhang AP, Yang S, Gao M, Wei SC, He PP *et al.* (2003) Association of HLA class I alleles with psoriasis vulgaris in southeastern Chinese Hans. *J Dermatol Sci* 33:1–6

Possible Involvement of Exon 31 Alternative Splicing in Phenotype and Severity of Epidermolysis Bullosa Caused by Mutations in *PLEC1*

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TO THE EDITOR

Epidermolysis bullosa constitutes a group of phenotypically diverse genodermatoses which manifest with blistering and erosions of the skin and mucous membranes (Fine *et al.*, 2000). Recent advances in epidermolysis bullosa research have allowed the identification of mutations in 10 different genes, which account for the clinical heterogeneity in epidermolysis bullosa (Pulkkinen and Uitto, 1999).

Mutations in the plectin gene (*PLEC1*) are generally thought to cause

Abbreviations: EBS, epidermolysis bullosa simplex; EBS-MD, epidermolysis bullosa simplex associated with muscular dystrophy; EBS-PA, epidermolysis bullosa simplex associated with pyloric atresia; PTC, premature termination codon

epidermolysis bullosa simplex (EBS) associated with muscular dystrophy. The majority of these cases are characterized by generalized blistering and muscular dystrophy (McLean *et al.*, 1996; Smith *et al.*, 1996; Shimizu *et al.*, 1999). Muscle weakness is first observed during the latter part of the first decade of life. However, we and other groups have recently demonstrated that lethal EBS cases with pyloric atresia (EBS-PA) also result from mutations in *PLEC1* (Nakamura *et al.*, 2005; Pfendner and Uitto, 2005b). Seven cases of this new variant of EBS have been reported so far (Pfendner *et al.*, 2005a). These patients manifest with cutaneous blisters, aplasia cutis congenital (severe localized absence of skin), and pyloric atresia, which rapidly result in the patient's demise, often soon after birth. This study reports two cases with defective plectin expression that show EBS-PA and EBS with muscular dystrophy (EBS-MD). Furthermore, based on data mining from the *PLEC1* mutation database, we suggest the possible involvement of exon 31 in alternative splicing that may alleviate the phenotypic severity of epidermolysis bullosa cases caused by mutations in the plectin gene.

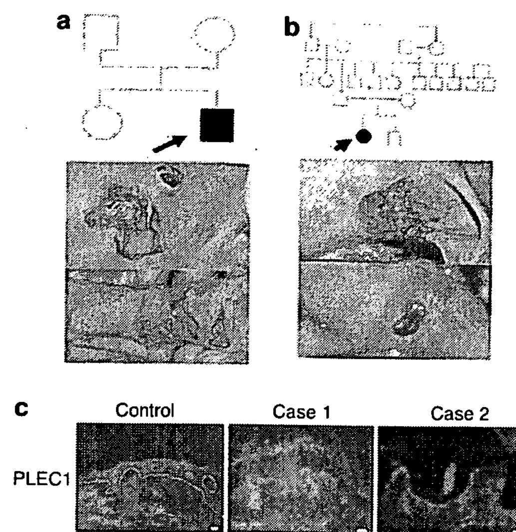

Figure 1. Family trees, clinical findings and plectin expression. (**a**) Case 1 was a 4-month-old boy who has exhibited skin fragility since birth. There was no family history of skin fragility. Blisters and erosions were scattered over his whole body and oral mucous membranes were also affected. Healing occurred without scarring and milia formation. He was diagnosed as suffering from pyloric atresia by routine abdominal X-ray. On the fourth day after birth, pyloroplasty was performed. He suffered from aspiration pneumonia and impairment of weight gain before the age of 6 months, but afterwards the volume of milk taken increased and blister formation steadily lessened. No muscular and neurological findings were observed. (**b**) Case 2 was a 49-year-old female with skin fragility from birth. Several blisters and erosions were found on her trunk and extremities. Slight scar formation was seen in some areas. Hypoplasty of her permanent dentition was seen and some nail thickening was observed. Her family tree showed a history of consanguinity. Although mild blister formation continued, muscle symptoms had never been found, until muscle weakness on the arms was first noted at the age of 19 years. Muscle weakness gradually progressed, but she was able to perform routine activities. However, she could not walk at the age of 38 years, owing to widespread muscular atrophy. She is now confined to a bed and breathing is assisted by a respirator. (**c**) Direct immunofluorescence analysis using mAb HD1-121 against plectin (a kind gift from Dr Owaribe K, Nogoya University) demonstrated that immunoreactivities were markedly attenuated in cases 1 and 2 compared with normal control. Bar = 50 μm.

Case 1 was a 4-month-old boy with skin fragility from birth. There was no other family history of skin fragility (Figure 1a). Generalized blisters and erosions were found over his entire body. He was diagnosed as suffering from pyloric atresia by routine abdominal X-ray. Case 2 was a 49-year-old female with skin fragility from birth (Figure 1b). Family tree showed a history of consanguinity, although there was no other family history of skin fragility. She is now bedridden and requires a respirator owing to progressive muscular dystrophy. Some blisters and erosions were observed on her trunk and extremities.

An immunohistochemical study using mAbs to a range of basement membrane zone component proteins was performed. Immunoreactivity against plectin rod domain was markedly attenuated in cases 1 and 2 (Figure 1c). Immunostaining for other basement membrane zone proteins including bullous pemphigoid antigens 1 and 2, the $\alpha6$ and $\beta4$ integrins, laminin 5, and type VII collagens were normal (data not shown). Direct nucleotide sequencing of *PLEC1* demonstrated that case 1 harbored novel heterogeneous premature termination codon (PTC) mutations Q2466X in exon 31 and Q2545X in exon 32, whereas case 2 harbored a novel homozygous PTC mutation, Q1450X, in exon 31 (Figure 2a). Informed consent was obtained from all individual subjects in this study. The protocols were approved by the Ethical Committee at Hokkaido University Graduate School of Medicine. This study was conducted according to the Declaration of Helsinki Principles.

Cases 1 and 2 demonstrated extracutaneous involvement including PA and MD, respectively. We believe that the clinical features and course of case 2 were typical of EBS-MD. Although all seven previous cases of EBS-PA showed a severe, lethal clinical course (Pfendner *et al.*, 2005a), case 1 was much milder than those cases and even showed some clinical improvement over time, so this is the first case of EBS-PA with a relatively moderate phenotype.

The precise pathomechanism causing the clinical differences between EBS-MD and EBS-PA has not yet been elucidated (Nakamura *et al.*, 2005; Pfendner *et al.*, 2005a; Pfendner and Uitto, 2005b). The *PLEC1* mutation database has accumulated almost 40 *PLEC1* mutations from 22 cases of EBS-MD and seven cases of EBS-PA (Pfendner *et al.*, 2005a, McMillan *et al.*, 2007). We have carefully re-examined genotype–phenotype correlations in EBS-MD and EBS-PA. The plectin database contains many homozygous mutations and we plotted only homozygous PTC mutations in order to minimize the effect of the expression difference

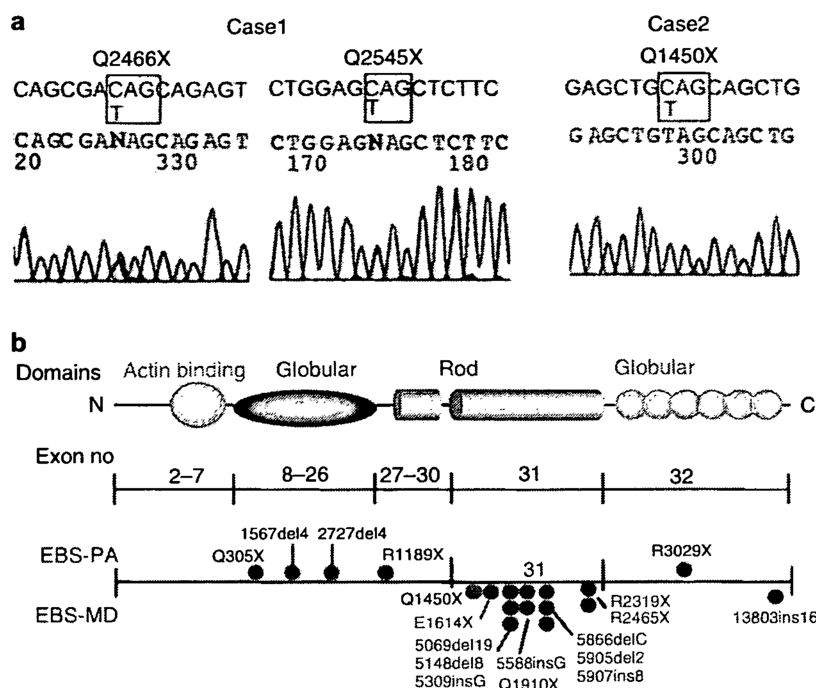

Figure 2. *PLEC1* mutations and significance of exon 31. (**a**) Genomic DNA was obtained from both cases and the parents. The mutation detection strategy was performed after PCR amplification of all exons and intron–exon borders, followed by direct automated nucleotide sequencing. The genomic DNA nucleotides, the complementary DNA nucleotides and the amino acids of the protein were numbered based on the previous sequence information (GenBank accession no. AH003623) (McLean *et al.*, 1996). Case 1 demonstrated heterozygous nonsense mutations. The maternal nonsense mutation was a C →T transition at nucleotide c.7396 of complementary DNA in exon 31, resulting in the substitution of a glutamine (CAG) at position 2466 with a stop codon (TAG) (Q2466X). The other paternal nonsense mutation was also a C→T transition at nucleotide c.7633 of complementary DNA in exon 32, resulting in the substitution of a glutamine (CAG) at position 2545 with a stop codon (TAG) (Q2545X). Case 2 showed homozygous nonsense mutations, which was a C→T transition at nucleotide c.4348 of complementary DNA in exon 31, resulting in the substitution of a glutamine (CAG) at position 1450 with a stop codon (TAG) (Q1450X). (**b**) The plectin database shows many homozygous mutations and we plotted the position of only homozygous PTC mutations. Interestingly, homozygous PTC mutations associated with EBS-MD (blue circles) are located in exon 31 except for one mattation13803ins16 whereas those with EBS-PA (red circles) are in other parts of the gene except exon 31. Q1450X (green circle) is the present case. Amino-terminal actin binding domain, amino-terminal globular domain, rod domain and carboxyl-terminal globular domain are shown. Positions of exons are indicated by numbers (exon no).

between the two *PLEC1* alleles. Interestingly, homozygous PTC mutations associated with EBS and MD are located in exon 31 except for one (13803ins16), whereas those with EBS with PA are located in parts of the gene other than exon 31 (Figure 2b). Thus, the plectin database suggested that EBS-MD and EBS-PA were associated with mutations in exon 31 and other than in exon 31, respectively.

Analysis of the murine plectin gene showed that alternative splicing resulted in more than 16 plectin variants and that tissue-specific expression of these variants was different (Fuchs *et al.*, 1999; Rezniczek *et al.*, 2003). This leads to the possibility that PLEC1 alternative splicing affects the severity of blistering and extracutaneous manifestation. Plectin comprises a central rod domain with a α-helical coiled-coil structure and large flanking amino-and carboxyl-terminal globular domains (Liu *et al.*, 1996; Wiche, 1998). Recently, one alternate splice messenger RNA transcript which lacks exon 31 encoding the central rod domain was identified in multiple rat tissues (Elliott *et al.*, 1997; Steinboeck and Kristufek (2005)). In fact, skin fragility in patients with *PLEC1* mutations was less severe than that observed in plectin-deficient mice. As most of *PLEC1* mutations are located within the rod domain that was not present in the smaller splice variant, this variant might in part compensate for the loss of the canonical (full length) plectin expression in humans (Litjens *et al.*, 2006). To understand the expression of the rodless alternative spliced form in various human tissues and cells, we performed plectin domain-specific RT-PCR, which indicated that human cells also express the rodless isoform at various levels (data not shown).

A combination of our mutations and plectin expression results with the above mutation database, suggests that exon 31 alternate splicing may restore the *PLEC1* open-reading frame in EBS-MD patients with PTC mutations in exon 31, and partially rescue the phenotype. Therefore, we suggest that EBS-MD caused by mutations in exon 31 demonstrates a milder phenotype than EBS-PA caused by non-exon 31 *PLEC1* mutations. Furthermore, the relatively moderate phenotype of case 1 with non-lethal EBS-PA might result from one mutation associated with exon 31.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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