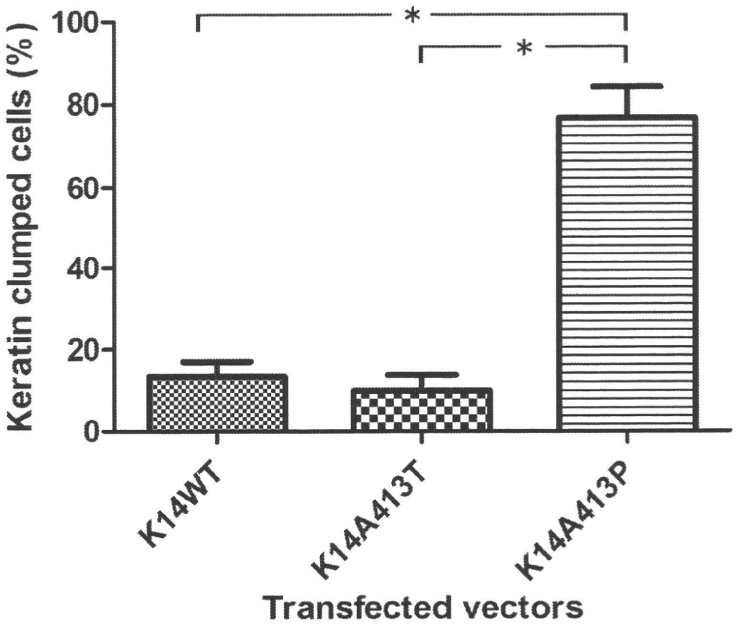
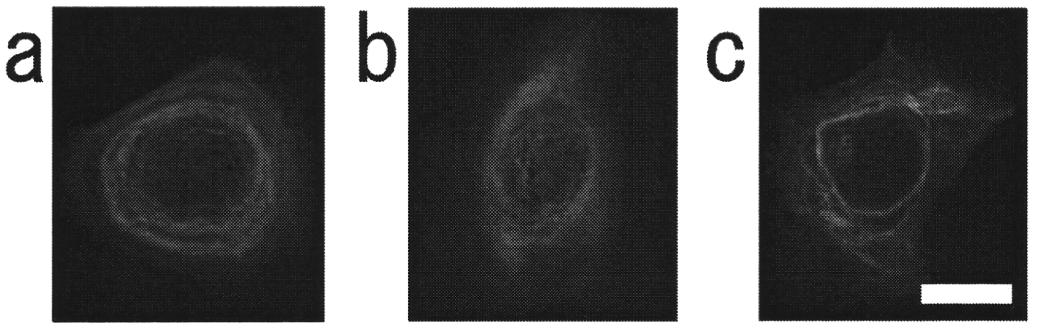


Supplementary Figure 2. *In vitro* assay using HeLa cells transfected with mutated *KRT14* cDNA

The percentage of cells showing keratin aggregates among transfected HeLa cells is compared. Each value shown represents the mean ± SEM of ten individual samples. There are significantly more keratin clumped cells observed in the K14A413P-transfected HeLa cells (77±7%) than in those transfected with either K14WT (14±4%) or K14A413T (10±4%). The statistical significance of the differences between groups is assessed by one-way ANOVA followed by Tukey's test (*, $p < 0.05$).



Supplementary Figure 3. *In vitro* assay using normal human epidermal keratinocytes transfected with mutated *KRT14* cDNA

Normal human epidermal keratinocytes (NHEK) transfected with K14WT (a) or K14A413T (b) or K14A413P (c) (bar: 5 μm). No keratin aggregates were observed in any of the groups.

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

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Letter to the Editor

Sjögren-Larsson syndrome (SLS) is a rare, autosomal recessive neurocutaneous disorder characterized by clinical triads, congenital ichthyoids, spasticity and mental retardation [1]. SLS is caused by mutations in fatty aldehyde dehydrogenate *(FALDH)* (or *ALDH3A2)* gene [1]. FALDH is a microtonal NAD-dependent enzyme, which oxidizes medium- to long-chain aliphatic aldehydes to fatty acids. Accumulation of fatty alcohol has been shown in cultured fibroblasts and in plasma from SLS patients [1]. Numbers of mutations of *FALDH* gene have been shown, although only three mutations have been identified in Japanese SLS patients [2, 3, 4]. We here report a SLS patient who is a homozygote for one of the known mutations. In addition to assessing skin phenotype, permeability barrier function and cutaneous morphology, biochemical analysis revealed novel alterations in lipid profiles in the stratum corneum associated with barrier function.

A 57-year-old Japanese woman complaining of slightly purity and dry skin with scaling visited our hospital. The patient has been suffering from scaly skin lesions over the entire body since her early childhood. She presented generalized dryness,

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

Transepidermal water loss (TEWL) of the ichthyosiform lesion on the extensor and flexor sides of the forearm and back (6.3, 12.2, 10.2 g $h^{-1}m^{-2}$, respectively) was

within the normal range (0-10, very good; 10-15, good; 15-20, fair; 25-30, poor; more than 30, very poor). On the other hand, water retention capability was impaired in the lesion (25.5, normal >60).

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

The identical mutation in our case was described in another Japanese patient with SLS [2]. The other mutations reported in the Japanese cases were c.481delA,

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

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

It remains to be resolved, however, why FFA level was high in spite of the deficient activity of FALDH, which is the enzyme catalyzing the sequential oxidation of fatty alcohol to fatty acid. It is likely that increased levels of wax esters and

alkyl-diacylglycerol in scales and keratinocytes of SLS [9] derived from fatty alcohol may contribute to FFA production via hydrolysis with lipase, because the levels of these lipids were high.

Consistent with a prior study showing a deficiency of Cer 1, 6 in SLS patients' skin [10], Cer 1, 6, 7 were decreased in the epidermis of our case. We further demonstrated that the levels of CLE-bound ceramides, Cer A, which are produced from acylglucosylceramide, elevated in the scale from the patient, although Cer 1 (EOS) generated from the same precursors decreased. Therefore, acylglucosylceramides appear to be preferentially utilized for CLE-bound ceramide production rather than free (CLE-unbound) lipid production in the SC. Exact mechanisms for CLE formation have not been elucidated yet and it remains to be resolved whether preferential utilization of acylglucosylceramide for CLE formation occurs only in the present case or also in other SLS patients. Moreover, it is unknown how decrease in Cer 1, 6, 7 occur and whether barrier lipid abnormality in the patient was a primary event or a secondary phenomenon in the pathogenesis of SLS skin lesions. Cer 1 is essential lipid species to form epidermal permeability barrier formation. Thus, not only accumulation of free

fatty acids, but also deficiency of specific creamed species might contribute to formation of ichthyotic phenotype in SLS.

We greatly thank Drs Sumiko Hamanaka (), Ken Hashimoto (Department of Dermatology, Wayne State University School of Medicine), for helpful discussion.

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Figure legends

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

Fig 2. (a) Sequencing analysis of FALDH gene. A homozygous point mutation (c.1157A>G) in the exon 8 that substitutes asparagine for serine at position 386 (p.Asn386Ser). (b) Lipid analysis of scales taken from sunburn lesions of a normal control individual (white bar) and from the patient's lesions (black bar) show increased FFA and Cer A level and decreased ceramide 1, 6, 7 levels in the patient's scale compared with control samples. Scales were taken from the upper back skin of the patient or control subjects. Gene and lipid analysis were performed as we described previously [5, 6].

Figure 1

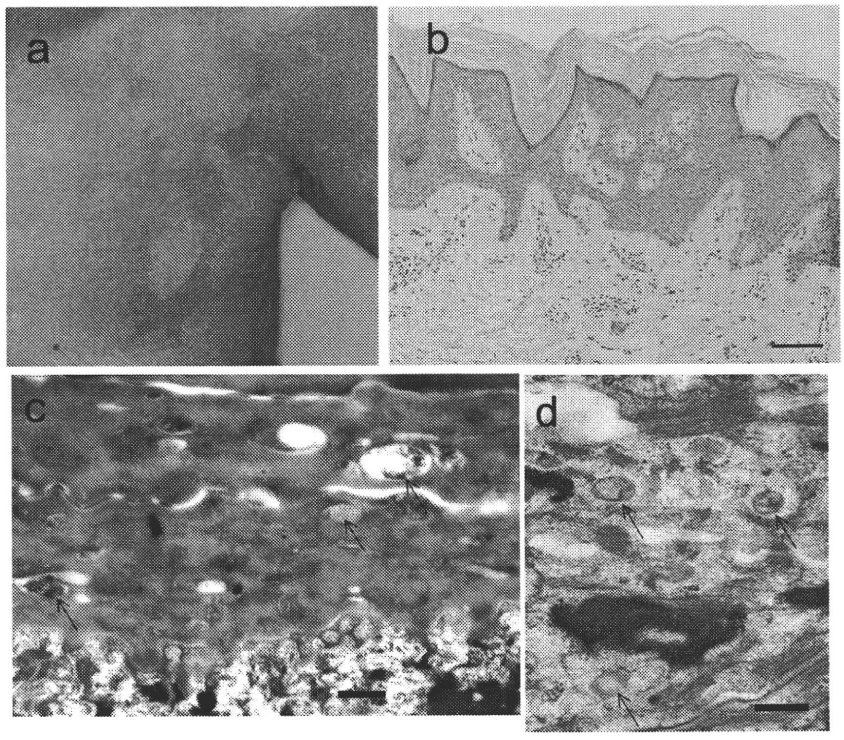
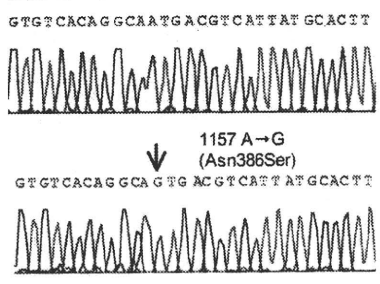


Figure 2



Letter

Case letter

Extremely severe palmoplantar hyperkeratosis in a generalized epidermolytic hyperkeratosis patient with a keratin 1 gene mutation

To the Editor: Epidermolytic hyperkeratosis (EHK; OMIM#113800), also called bullous congenital ichthyosiform erythroderma, is a rare genetic disorder of keratinization. We report a patient with generalized EHK showing extremely severe palmoplantar hyperkeratosis with digital contractures.

A 45-year-old Japanese man had erythroderma at birth. He exhibited skin blistering, erosions, and hyperkeratosis on the erythrodermic skin since infancy. The blistering and erosions gradually diminished with age. He developed severe palmoplantar hyperkeratosis and digital contractures at 7 years of age. At 24 years of age, surgery was performed to improve the contraction of his toes. A physical examination revealed hyperkeratosis of the entire body, especially at the ankles, elbows, and knees, and erosions were observed on the inner side of the elbows and knees (Fig 1, *A-D*). Palmoplantar hyperkeratosis was severe with digital contractures. The morphology of his hair, nails, and teeth was normal. There was no known family history of skin disease. Skin biopsy from the upper portion of the left arm showed severe granular degeneration in all the suprabasal layers (Fig 1, *E*). Ultrastructural analysis revealed clumping of the intermediate filaments within keratinocytes of the suprabasal layers (Fig 1, *F*).

Direct sequencing of the whole coding regions of *KRT1* and *KRT10* (GenBank accession numbers NT029419.11 and NT010755.15) was performed as previously described,[1] and a novel heterozygous *KRT1* missense mutation c.1457T>G (p.Leu486Arg) was identified in exon 7. This mutation was verified by restriction enzyme *Msp*I digestion. The mutation p.Leu486Arg was not found in 100 normal, unrelated Japanese alleles (50 healthy unrelated individuals) using sequence analysis (data not shown).

The present novel *KRT1* mutation p.Leu486Arg is in the 2B segment of keratin 1 (Fig 2, *A* and *B*). This mutation occurred within the highly conserved helix termination motif (HTM) of the K1 protein. The palmoplantar hyperkeratosis was extremely severe. It is noteworthy that another mutation at the identical

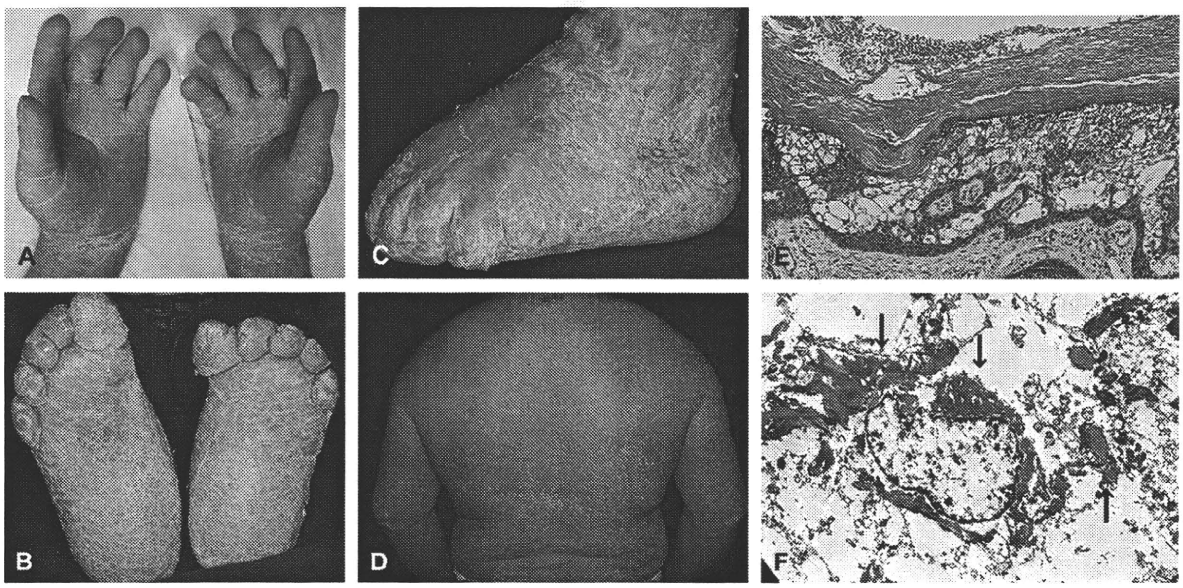


Fig 1. Clinical, histopathologic, and ultrastructural features of the patient. Severe diffuse hyperkeratosis and scale are seen on the palms (**A**) and soles (**B**). Warty brown hyperkeratosis and scale are present on the margins and the dorsal surface of the foot (**C**). Generalized erythroderma and scaling is seen on the trunk (**D**). The histopathologic examination revealed acanthosis and hyperkeratosis, coarse keratohyaline granules, and severe granular degeneration in the entire spinous and granular layers of the epidermis (**E**). Ultrastructurally, clumping of the keratin filaments *(arrows)* is seen within an upper epidermal keratinocyte (**F**).

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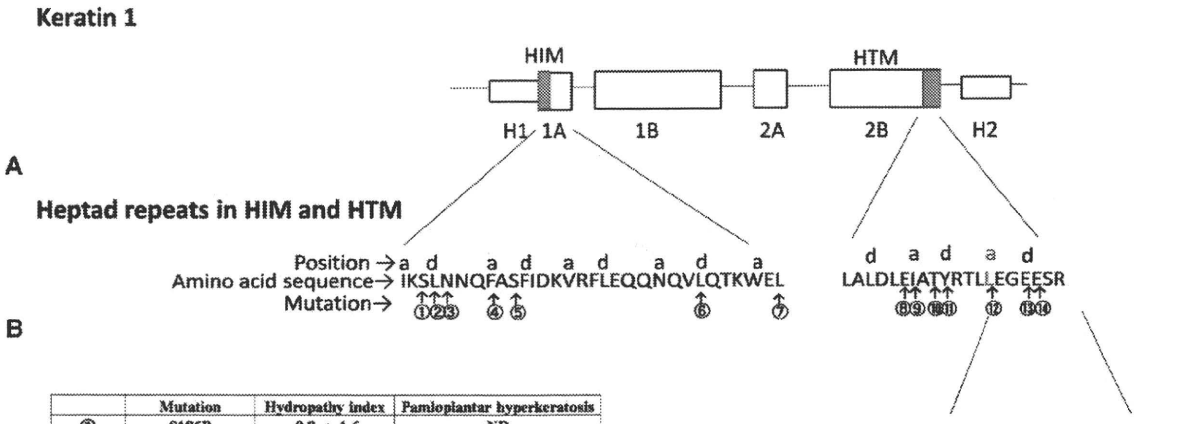

C

	Mutation	Hydropathy index	Palmoplantar hyperkeratosis
①	S186P	-0.8→-1.6	ND
②	L187F	3.8→2.8	mild
②	L187F	3.8→2.8	mild
③	N188S	-3.5→-0.8	ND
③	N188S	-3.5→-0.8	severe
③	N188S	-3.5→-0.8	severe
③	N188K	-3.5→-3.9	severe
③	N188T	-3.5→-0.7	ND
④	F191I	2.8→4.5	mild
④	F191C	2.8→2.5	severe
⑤	S193P	-0.8→-1.6	severe
⑥	L208P	3.8→-1.6	severe
⑦	L214P	3.8→-1.6	mild
⑧	E478K	-3.5→-3.9	mild
⑧	E478D	-3.5→-3.5	mild
⑨	I479F	4.5→2.8	mild
⑨	I479F	4.5→2.8	mild
⑨	I479T	4.5→-0.7	mild
⑨	I479T	4.5→-0.7	mild
⑩	T481P	-0.7→-1.6	severe
⑪	Y482C	-1.3→2.5	mild
⑫	L486P	3.8→-1.6	severe
⑫	L486R	3.8→-4.5	severe
⑬	E489K	-3.5→-3.9	mild
⑭	E490Q	-3.5→-3.5	mild
⑭	E490G	-3.5→-0.4	mild

ND, not described.

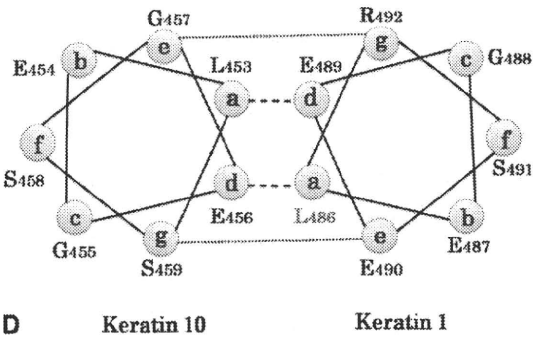

Fig 2. Summary of mutations in the helix initiation motif (HIM) and helix termination motif (HTM) of K1 from the Human Intermediate Filament Database (www.interfil.org/). **A**, Molecular structure of K1. **B**, Heptad repeats in HIM and HTM of K1 and mutation sites. The majority of cases (22 of 26) had mutations in the heptad repeat position *a, d, e,* and *g*. The present mutation is located at the *a* position leucine residue at codon no.486 *(red characters)* in the C-terminal–most heptad repeat. **C**, Summary of the *KRT1* mutations in HIM and HTM, alterations of hydropathy index, and levels of palmoplantar hyperkeratosis. Eight cases, including the present one, were reported as showing severe palmoplantar hyperkeratosis, and seven of those nine patients harbored mutations in the important *a, d, e,* and *g* position of heptad repeats. Mutations in this 486-leucine residue may seriously perturb the stability of keratin intermediate filaments. The substitution of arginine for leucine alters the character of the amino acid from that of a hydrophobic, apolar amino acid (hydropathy index of leucine, +3.8) to that of the most hydrophilic, basic amino acid (hydropathy index of arginine, −4.5). **D**, Heptad structure of the rod domain: schematic of a transverse cut through the last heptad (*abcdefg*) of the HTM of K1 and K10, showing hydrophobic interactions between positions *a* and *d (dashed lines)* and ionic hydrogen interactions between positions *e* and *g (dotted lines)*. Position *a* is occupied by apolar, hydrophobic amino acids. The *a* residues are thought to interact with amino acids located in the *d* position of the partner molecule of the heterodimer through hydrophobic interactions which stabilize the two-chain coiled-coil molecules. When the two strands coil around each other, positions *a* and *d* are internalized, stabilizing the structure, while positions *b, c, e, f,* and *g* are exposed on the surface of the protein. Residues at positions *e* and *g* stabilize dimer formation through ionic and hydrogen bonds.

position of K1, p.Leu486Pro, was reported in patients with EHK and severe palmoplantar hyperkeratosis (Fig 2, *C*) and digital contractures, and the affected individuals exhibited clinical features similar to our patient.[2] Therefore, our data suggest that a nonconservative amino acid change at codon 486 of K1 results in a severe form of generalized EHK.

The rod domains consist of four alfa-helical segments that possess a repeating heptad amino acid residue peptide motif *(a-b-c-d-e-f-g)n* that has the potential to form a two-chain coiled coil with a corresponding sequence (Fig 2, *D*).[3-5] The residues at position *a, d, e,* and *g* are considered to be highly sensitive to mutations.[6]

Our patient with generalized EHK had most severe palmoplantar hyperkeratosis compared to previously reported cases with mutations in *KRT1*. The leucine residue at codon 486 is located in the *a* position of the heptad repeat at the C-terminal end of the 2B helix, and the substitution of arginine for leucine seriously alters the character of amino acid. It is therefore reasonable to say that this mutation caused generalized EHK with severe palmoplantar hyperkeratosis, compared with that seen in patients harbouring mutations in the other residues.

Twenty-six EHK cases, including the present case with point mutations at the helix initiation motif (HIM) and HTM of *KRT1*, have been reported to date (Fig 2, *C*); Human Intermediate Filament Database [www.interfil.org/]). Only nine cases, including the present case, were diagnosed as generalized EHK with severe palmoplantar hyperkeratosis, and seven cases out of nine harbored missense mutations in the heptad repeat position *a, d, e,* and *g*. These facts indicate that the mutation site and the nature of amino acid alterations in K1 may determine the level of severity of palmoplantar hyperkeratosis.

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Conflicts of interest: None declared.

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Recurrence of Hydroxyurea-induced Leg Ulcer After Discontinuation of Treatment

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Hydroxyurea (HU) is a hydroxylated derivative of urea that has been recognized since 1960 as effective against cancer (1). It is an inhibitor of cellular DNA synthesis, and it promotes cell death in the S phase of the cell cycle through inhibition of the enzyme ribonucleotide reductase (2). The most common indications for HU therapy are chronic myeloid leukaemia and other myeloproliferative disorders (3, 4) such as essential thrombocythemia (5) and polycythemia vera (PV) (6). Cutaneous side-effects, such as alopecia, diffuse hyperpigmentation, scaling, lichen planus-like lesions, poikiloderma, atrophy of the skin and subcutaneous tissues, and nail changes, can occur during the treatment with HU (7–9). The occurrence of painful leg ulcers represents another rare and incompletely characterized complication that has been described in patients with myeloproliferative diseases receiving high-dose long-term HU treatment (10). While the mode of action of HU on bone marrow elements is well established, its effects on actively proliferating epithelial cells remain less described (11). Poor response to traditional local and systemic therapy is a typical feature of HU-induced leg ulcers, and discontinuation of the drug is often required to achieve complete wound healing (6, 8). Cessation of the drug usually improves the skin ulcer; although, in some cases, the ulcer remains and additional therapies, such as skin grafting, are needed (12). We report here the first case of a leg ulcer that recurred even after discontinuation of HU treatment.

CASE REPORT

The patient was an 82-year-old Japanese male who had been diagnosed with PV 9 years before and had been treated only with phlebotomy and an anti-platelet agent for several years. Due to splenomegaly and elevated blood cell counts, HU therapy was started 3 years ago at a dosage of 1 g daily for a month, followed by 1.0 or 1.5 g daily for 28 months. A good clinical response was achieved. However, the patient developed painful ulcers on the left second toe after two years of HU treatment.

He visited our outpatient clinic and was diagnosed with an HU-induced skin ulcer. HU was discontinued, topical application of sulfadiazine silver was performed, an oral antibiotic (cefdinir) was administered, and the ulcer epithelialized. However, a new ulcer appeared on the left lateral malleolar area 46 days after cessation of HU and gradually enlarged in size. The patient was admitted to our hospital for treatment of the ulcer.

Examination revealed a 48 × 56 mm ulcer with yellow necrotic tissue and marginal erythematous oedema (Fig. 1). Laboratory examination revealed a white blood cell count of $11.6 \times 10^3/\mu l$, a platelet count of $64.2 \times 10^4/l$, and a red blood cell count of $5.07 \times 10^6/\mu l$. Anti-nuclear antibody, anti-neutrophilic cytoplasmic antibodies, anti-cardiolipin antibody, and cryoglobulin were negative. A skin biopsy taken from the margin of the ulcer demonstrated leukocytoclastic vasculitis in the upper dermis (Fig. 2). A wound-healing strategy of surgical debridement, intravenous prostaglandin E1 administration, and topical application of beta-fibroblast growth factor, sulfadiazine silver and alprostadil alfadex was started, and the ulcer began to epithelialize. After 4 months, re-epithelialization was complete. The PV was treated with busulfan, achieving a good clinical response.

DISCUSSION

HU is usually well tolerated and has low toxicity (1). However, cutaneous adverse effects such as diffuse hyperpigmentation, brown discoloration of the nails, acral erythema, photosensitization, fixed drug eruption, alopecia, and oral ulceration have been reported (7–9). Stahl & Silber (10) first reported HU-induced skin ulcers in 1985. Montefusco et al. (11) reported

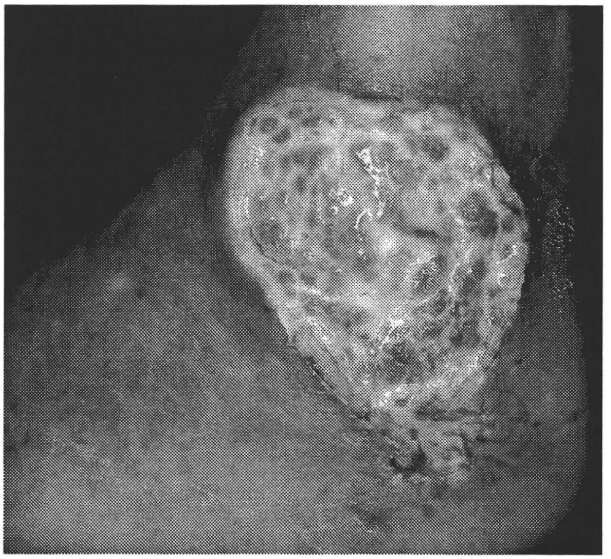

Fig. 1. Left foot with an ulcer on the lateral malleolar area after two months free of hydroxyurea administration. The ulcer was covered with yellow necrotic tissue and surrounded by oedematous erythema.

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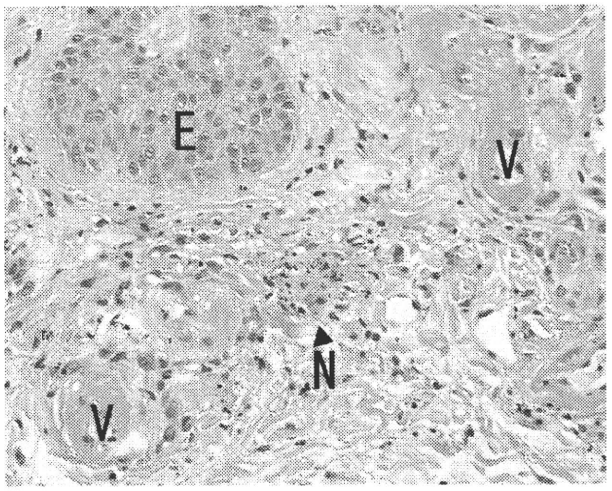

Fig. 2. Histology of erythema on the margin of the ulcer (haematoxylin-eosin staining). Fibrin deposition on the vascular wall and nucleic debris were evident around small vessels (×100). (E: epidermis; V: blood vessels; N: neutrophilic nuclear debris)

that, among 200 chronic myeloid leukaemia patients treated with HU, 17 (8.5%) developed leg ulcers. However, they achieved complete resolution or significant improvement after discontinuation of HU therapy (11). HU-induced leg ulcer and complete resolution within several months after drug discontinuation has also been reported in other myeloproliferative disorder, such as PV (6) and essential thrombocythemia (5). In those cases, as in ours, most of the patients had been treated with > 1 g of HU per day for at least one year (8). In the present case, the patient was treated with > 1 g of HU per day for 28 months. The ulcer occurred on his lateral malleolus, which histologically showed leukocytoclastic vasculitis. These features are consistent with previous reports of HU-induced leg ulcer.

From previous reports, the pathogenesis of HU-induced ulceration remains unclear and it may be multifactorial. It has been postulated that ulcers may be the result of: (*i*) interruption of microcirculation due to leukocytoclastic vasculitis or arterial microthrombi related to platelet dysregulation (13, 14); (*ii*) cumulative toxicity in the basal layer of the epidermis through inhibition of DNA synthesis (8); and (*iii*) repeated mechanical injury in areas subject to trauma: a perimalleolar area for instance (15).

In the case described here, a new ulcer developed even after cessation of HU administration. As for the pathogenic mechanism of recurrence, (*i*) interruption of microcirculation could result from hyperviscosity due to the elevated platelet count (as high as 100×10^4/l in one measurement) (13, 14), although no thrombi were observed histologically in the capillaries or small vessels. (*ii*) The direct cytotoxic effect of HU (8) may continue even after the withdrawal of the drug, and it may inhibit the repair of (*iii*) small injuries in the perimalleolar area: the one of the area susceptible to physical trauma (15). These assumptions can be made from the pathogenesis of HU-induced ulcer reported previously (8, 13–15).

To our knowledge, this is the first report of recurrence of HU-related leg ulcer after the discontinuation of medication. The case suggests that it is important to pay careful attention to recurrence even after cessation of HU therapy. Precise, early treatment for microtraumas and small ulcers should be administered to patients with a long history of HU medication.

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

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Abstract

Background Mutations in the plectin gene (*PLEC*) generally lead to epidermolysis bullosa simplex (EBS) associated with muscular dystrophy. It has been recently demonstrated that *PLEC* mutations can also cause a different clinical subtype, EBS associated with pyloric atresia (EBS-PA), which shows early lethality. Prenatal diagnosis (PND) of EBS-PA using mutation screening of *PLEC* has not been described.

Objective This study aimed to perform DNA-based PND for an EBS-PA family.

Materials and methods The EBS-PA proband was compound-heterozygous for a paternal c.1350G>A splice-site mutation and a maternal p.Q305X nonsense mutation. Genomic DNA was obtained from amniocytes taken from an at-risk fetus of the proband's family. Direct sequencing and restriction enzyme digestion of polymerase chain reaction products from the genomic DNA were performed.

Results Mutational analysis showed that the fetus harbored both pathogenic mutations, suggesting that the fetus was a compound-heterozygote and therefore affected with EBS-PA. The skin sample obtained by autopsy from the abortus confirmed the absence of plectin expression at the dermal–epidermal junction.

Conclusions This is the first successful DNA-based PND for an EBA-PA family.

Introduction

Epidermolysis bullosa (EB) comprises a group of diseases that are classified into four categories – EB simplex (EBS), junctional EB (JEB), dystrophic EB, and Kindler syndrome – depending on the depth of the dermal-epidermal junction split.[1] The four categories are subcategorized into minor subtypes, some of which show severe prognosis and lead to early demise.

Prenatal diagnosis (PND) of lethal EB subtypes has been performed for more than two decades. Electron microscopy and immunofluorescence analysis of fetal skin samples were the mainstay for PND of EB fetuses.[2] However, morphologically based PND had technical difficulties and abortion risks from the fetal skin biopsies. As the genes responsible for EB have been indentified, DNA-based PND has been available for many lethal EB subtypes.[2,3] Recently, other techniques such as immunofluorescence analysis of villous trophoblasts,[4] preimplantation genetic analysis[5], and preimplantation genetic haplotyping[6] have been described as useful for PND of EB.

Among the lethal EB subtypes, EB associated with pyloric atresia (EB-PA) has been known to result from mutations in the genes encoding either plectin (*PLEC*), or α6 (*ITGA6*), or β4 integrin (*ITGB4*).[1] EB-PA can either manifest as JEB with PA (JEB-PA) or EBS with PA (EBS-PA) and is categorized as hemidesmosomal variant of EB. EB-PA due to *ITGA6* or *ITGB4* mutations is generally characterized by blister formation at the level of the lamina lucida as JEB-PA, although skin separation within basal keratinocytes has been described in a few cases.[1] In contrast, it has been recently reported that another subset of lethal EB-PA shows an intraepidermal level of cleavage consistent with EBS, caused by mutations in the gene encoding plectin (*PLEC*).[7–9] To date, PND of EBS-PA using mutation screening of *PLEC* has not been reported in the literature. This paper describes the first DNA-based PND for an EBS-PA family.

Materials and Methods

The EBS-PA family

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

PND

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

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

Immunofluorescence analysis

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

Results

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

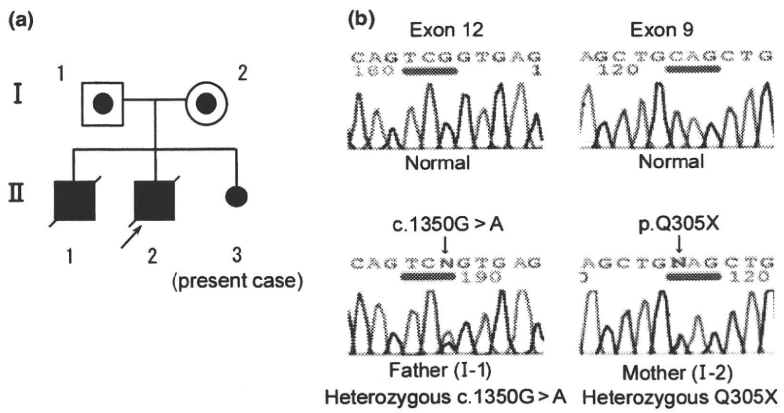

Figure 1 Family tree of the present case and the causative *PLEC* mutations. (a) The first and second newborns exhibited clinical features typical of EBS-PA and died shortly after birth. The proband (the second newborn) is indicated by an arrow. (b) The paternal splice-site mutation was a c.1350G>A transition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

