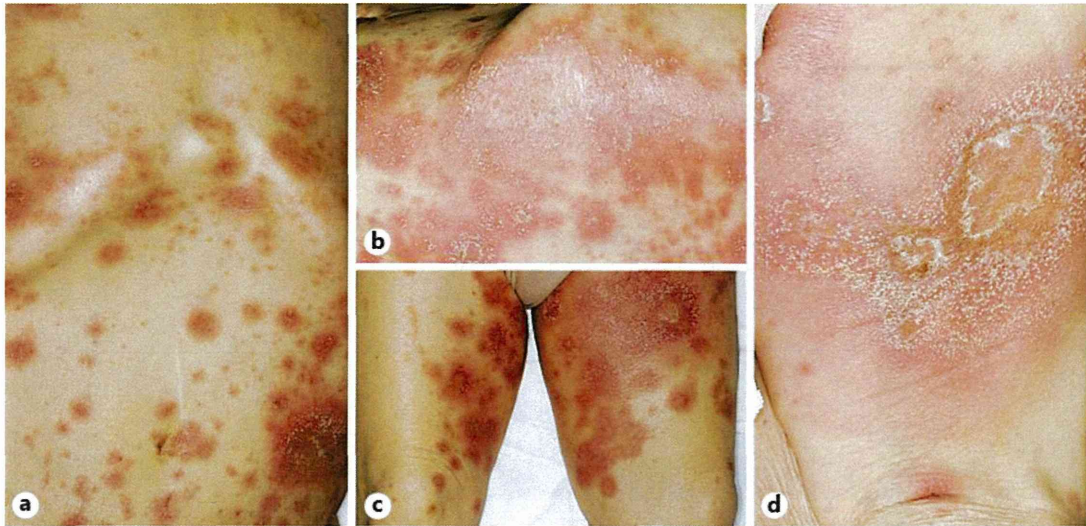**Fig. 2.** Clinical findings at his second visit. The patient came to our clinic because of high fever and general malaise. We found an extension and new development of erythema and pustules on the whole body. **a** Abdomen. **b** Buttock. **c, d** Thighs.
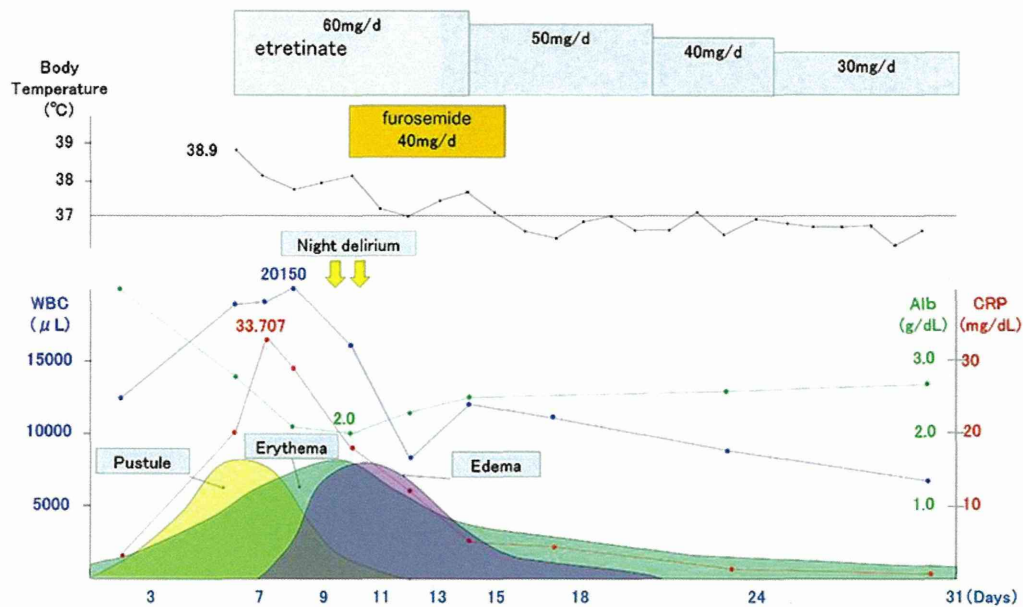

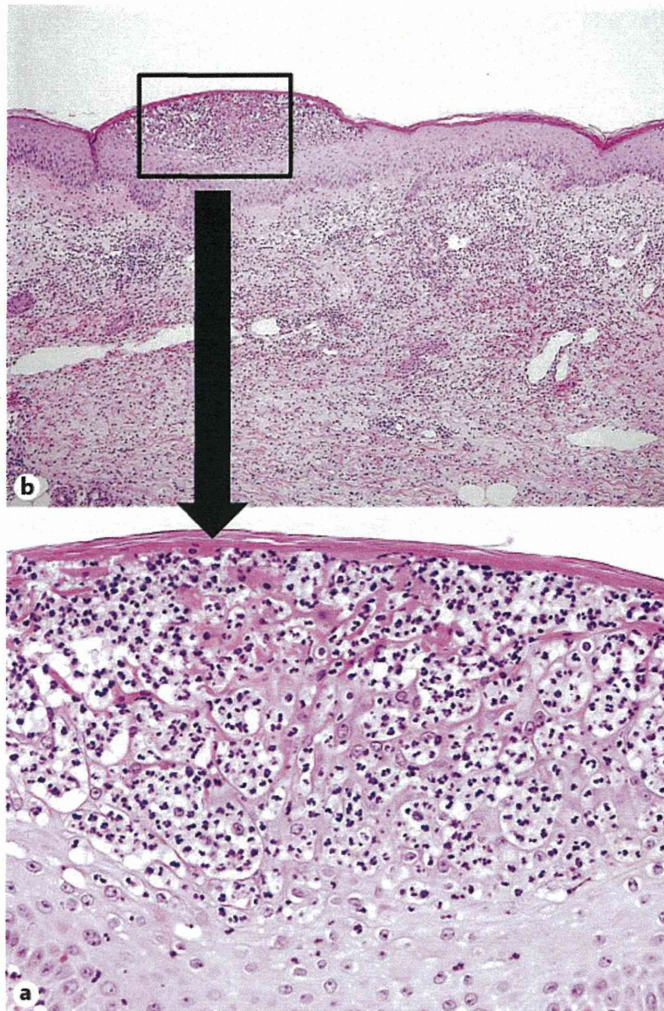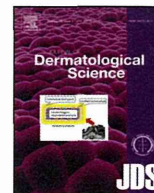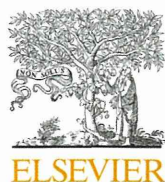
Fig. 3. Clinical course and treatments.

Fig. 4. a, b Histopathological findings. Neutrophil infiltration into the upper epidermis, which formed a spongiform pustule of Kogoj. **b** An enlarged photo of a part of **a. a** ×40. **b** ×200.

Invited review article

Update on autosomal recessive congenital ichthyosis: mRNA analysis using hair samples is a powerful tool for genetic diagnosis

CrossMark

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ABSTRACT

Research on the molecular genetics and pathomechanisms of autosomal recessive congenital ichthyosis (ARCI) has advanced considerably and several causative genes and molecules underlying the disease have been identified. Three major ARCI phenotypes are harlequin ichthyosis (HI), lamellar ichthyosis (LI), and congenital ichthyosiform erythroderma (CIE). Skin barrier defects are involved in the pathogenesis of ARCI. In this review, the causative genes of ARCI and its phenotypes as well as recent advances in the field are summarized. The known causative molecules underlying ARCI include *ABCA12, TGM1, ALOXE3, ALOX12B, NIPAL4, CYP4F22, PNPLA1, CERS3*, and *LIPN*. It is important to examine genetic associations and to elucidate the pathomechanisms of ARCI to establish effective therapies and beneficial genetic counseling. Next-generation sequencing is a promising method that enables the detection of causative disease mutations, even in cases of unexpected concomitant genetic diseases. For genetic diagnosis, obtaining mRNA from hair follicle epithelial cells, which are analogous to keratinocytes in the interfollicular epidermis, is convenient and minimally invasive in patients with ARCI. We confirmed that our mRNA analysis method using hair follicle samples can be applied not only to keratinization disorders, but also to other genetic diseases in the dermatology field. Studies that suggest potential next-generation therapies using ARCI model mice are also reviewed.

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Contents

1. Introduction

Autosomal recessive congenital ichthyosis (ARCI) is a heterogeneous group of keratinization disorders characterized primarily by abnormal skin scaling over the whole body [1]. These disorders are limited to the skin, and more than half of all patients present severe symptoms. Many patients are born as collodion babies (CBs). Later, the main symptoms are widespread scaling of the skin and varying degrees of erythema. ARCI includes a wide range of ichthyosis phenotypes. The main skin phenotypes are harlequin ichthyosis (HI, OMIM #242500), lamellar ichthyosis (LI, OMIM #242300), and congenital ichthyosiform erythroderma (CIE, OMIM #242100) [1].

The pathomechanisms and underlying genetic defects of ARCI have been elucidated recently. In addition, significant progress has been made toward understanding the molecular basis of human epidermal keratinization processes.

Since transglutaminase (TGase)-1 (*TGM1*) mutations were initially identified as the cause of LI in 1995 [2,3], several additional causal mutations have been detected in severe ARCI cases [4]. For instance, in 2005, we and other researchers reported loss-of-function mutations in the *ABCA12* gene in association with HI, the most severe type of ichthyosis [5,6].

To date, nine known genes are causally associated with ARCI in human patients [7], i.e., *ABCA12* [5,8–11], *TGM1* [2,3,12,13], the two lipoxygenase genes *ALOXE3* and *ALOX12B* [14,15], *NIPAL4* [16], *CYP4F22* [17,18], *PNPLA1* [19], *CERS3* [20,21], and *LIPN* [22] (Table 1). ARCI phenotypes exhibit a primary abnormality associated with barrier function in the stratum corneum as their underlying pathogenetic mechanism. The skin barrier of the stratum corneum has three major components, i.e., intercellular lipid layers, a cornified cell envelope, and keratin/filaggrin degradation products (Fig. 1).

In this review, we describe recent topics related to ARCI, highlighting the molecular mechanisms, causative genes, and clinical phenotypes of ARCI, and possible effective therapies. In addition, we briefly discuss mRNA analysis of hair samples, which is a minimally invasive method for mRNA analysis to aid in the genetic diagnosis of ARCI.

2. Causative ARCI genes and associated phenotypes

2.1. ABCA12

HI is a severe and often fatal congenital ichthyosis with an autosomal recessive inheritance pattern. It shows clinical features at birth including severe ectropion, eclabium, flattening of the ears, and large, thick, plate-like scales over the entire body. Neonatal death is not uncommon. HI is caused by severe functional defects in the keratinocyte lipid transporter ATP-binding cassette sub-family A
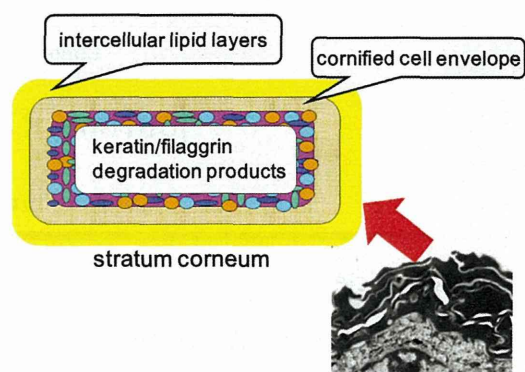

Fig. 1. Three major components of the stratum corneum that form the skin barrier. The major components are intercellular lipid layers, a cornified cell envelope, and keratin/filaggrin degradation products.

member 12 (ABCA12) (Fig. 2). ABCA12 functional defects lead to the disruption of lamellar granule (LG) lipid transport in the upper epidermal keratinocytes.

Mutations in *ABCA12* are known to cause not only HI, but also CIE and LI. In a review of the literature on *ABCA12* mutations in patients with ARCI, we identified 56 known *ABCA12* mutations in 66 globally distributed, unrelated families, including 48 HI, 10 LI, and 8 CIE families [23]. Of the 56 mutations, 36% are nonsense, 25% are missense, 20% are small deletion, 11% are splice-site, 5% are large-deletion, and 4% are insertion mutations. At least 62.5% of all reported mutations are predicted to result in truncated proteins. There is no obvious mutation hot spot in *ABCA12*, although mutations underlying the LI phenotype are clustered in the domain containing the first ATP-binding cassette.

Recent advances in HI research have revealed that high frequencies of HI can be improved with intensive neonatal care. Milstone and Choate [24] reported that the early introduction of overall intensive therapy might have contributed to better outcomes observed in HI babies who were given oral retinoids. Indeed, the outcomes of HI babies have recently improved in Japan. In a study of HI cases in Japan [25], we obtained clinical data for 16 HI patients between 2005 and 2010. Among them, there were 13 survivors (81.3%) and 3 deceased subjects (18.7%). Overall, we inferred that intensive neonatal care and, probably, early intervention using oral retinoids, have improved HI outcomes.

Recently, we identified the promoter region of *ABCA12* and located the essential elements therein; thus, the information necessary for genetic diagnostic screening for *ABCA12* mutations that cause congenital ichthyosis is available [26]. Specifically, we showed that ABCA12 expression in differentiating cultured human keratinocytes is critically dependent on a palindromic motif that resides in the region from −2084 to −2078 relative to the transcription start site (Fig. 3) [26].

Table 1
Causative genes and associated ARCI phenotypes.

| Gene	Protein	Disease
ABCA12	ATP-binding cassette sub-family A member 12	HI, LI, CIE
TGM1	Transglutaminase-1	LI, CIE, SHCB, Acral SHCB, BSI
ALOXE3	Lipoxygenase-3	LI, CIE, SHCB
ALOX12B	12(R)-lipoxygenase	LI, CIE, SHCB
NIPAL4	Nipa-like domain-containing 4	LI, CIE
CYP4F22	Cytochrome P450, family F polypeptide 2 homolog of leukotriene B4-omega-hydroxylase	LI
PNPLA1	Patatin-like phospholipase domain protein 1	LI
CERS3	Ceramide synthase 3	LI
LIPN	Lipase N	LI
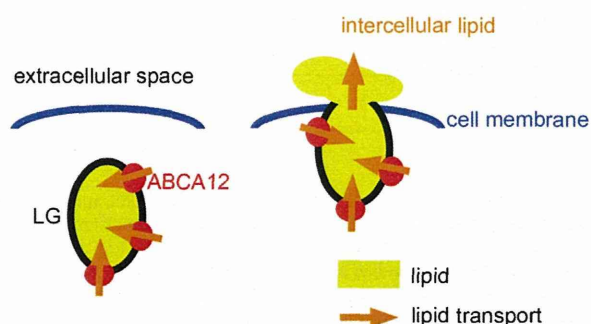
Fig. 2. Function of ABCA12. ABCA12 is a keratinocyte transmembrane lipid transporter protein associated with the transport of lipids by lamellar granules to the apical surface of granular layer keratinocytes. LG, lamellar granule.
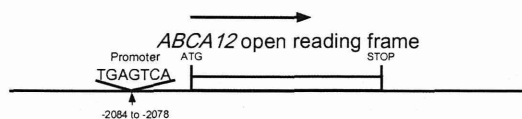
Fig. 3. Scheme of the *ABCA12* gene promoter. A palindromic motif (tgagtca) at −22084 to −22078 is essential for the promoter function of *ABCA12*.

As mentioned above, HI patients sometimes die during the perinatal period. However, most patients who survive beyond the neonatal period exhibit gradual phenotypic improvement from a few weeks to several months after birth [25]. To determine the mechanisms of phenotypic recovery, we investigated grafted skin and keratinocytes from *Abca12*-deficient (*Abca12*$^{-/-}$) mice [27]. We observed remarkable improvements for all if the abnormalities in the model mice with *Abca12*$^{-/-}$ skin grafts kept in a dry environment. The increased trans-epidermal water loss due to barrier defect was dramatically mitigated in the grafted *Abca12*$^{-/-}$ skin kept in a dry environment. An abnormal ceramide distribution, defective expression of differentiation-specific proteins, and profilaggrin/filaggrin conversion were observed in the primary culture of *Abca12*$^{-/-}$ keratinocytes, but these abnormalities were resolved in ten-passage sub-cultured *Abca12*$^{-/-}$ keratinocytes. These findings suggest that *Abca12*$^{-/-}$ epidermal keratinocytes restore normal differentiation during maturation. The precise mechanisms of this restoration of keratinocyte differentiation remain to be clarified, but the mitigation of keratinocyte differentiation defects may facilitate the development of novel therapies for the ichthyosis phenotype.

Cottle et al. [28] observed a pro-inflammatory signature characterized by chemokine upregulation in embryonic skin on *Abca12*-deficient mice. To examine the contribution of inflammation to disease development, they overexpressed interleukin-37b, an immunosuppressive interleukin, to globally suppress fetal inflammation. Considerable improvements in keratinocyte differentiation were observed in *Abca12*-deficient mice [28]. This result suggests a possible therapy for human ARCI involving *ABCA12* mutations.

2.2. TGM1

The cornified cell envelope on the inner surface of the cell membrane is essential for skin barrier function [29]. TGases in the epidermis are thought to be responsible, at least in part, for the assembly of cornified cell envelope precursor proteins, including involucrin, small proline-rich proteins, and loricrin, to form the cornified cell envelope [30]. The membrane-associated TGase-1 is the major TGase subtype expressed in the epidermis. Since the identification of TGase-1 (*TGM1*) mutations in a number of families with LI in 1995 [2,3], additional *TGM1* mutations associated with LI have been reported [12]. *TGM1* mutations have also been reported to underlie the CIE phenotype [31].

TGM1 mutations are not always related to severe phenotypes of ARCI. They sometimes cause very mild LI or self-healing collodion baby (SHCB), also termed self-improving collodion baby [32]. SHCB refers to a baby who is born as a collodion baby, but improves spontaneously. We recently identified missense mutations in the β-barrel domains of TGase-1 in arginine residues in very mild LI (Fig. 4A) and SHCB cases [12,33]. In LI, the majority of causative *TGM1* mutations have been identified in the central core domain or upstream of it. In contrast, mutations in β-barrel domains in the carboxyl-terminus of the gene are rarely found [7]. Several authors suggest that β-barrel domains increase TGase-1 activity, but are not essential for the basic function of the enzyme [8,9]. Clinically, other minor ARCI variants can be distinguished, e.g., bathing suit ichthyosis has been attributed to particular *TGM1* mutations that render the enzyme sensitive to ambient temperature. In acral SHCB, a very rare type, the collodion membrane is strictly localized to the extremities at birth and eventually resolves. This phenotype is also a result of *TGM1* mutations (Table 1) [1].

Recently, Aufenvenne et al. [34] have developed the basis for an enzyme-replacement therapy for individuals suffering from TGase1-deficient ARCI. They demonstrated that a topical approach using a formulation of liposomal-encapsulated recombinant human TGase-1 is suitable for restoring TGase-1 activity in the upper stratified layers of the epidermis to reconstitute epidermal integrity and barrier function in a mouse model. This result suggests a possible therapy for ARCI due to *TGM1* mutations.

2.3. ALOXE3 and ALOX12B

Mutations in two lipoxygenase genes, *ALOXE3* and *ALOX12B*, coding lipoxygenase-3 (eLOX-3) and 12(R)-lipoxygenase (12R-LOX), respectively, were reported to underlie ARCI in 2002 [14]. Oxygenation of the linoleate moiety of ceramides
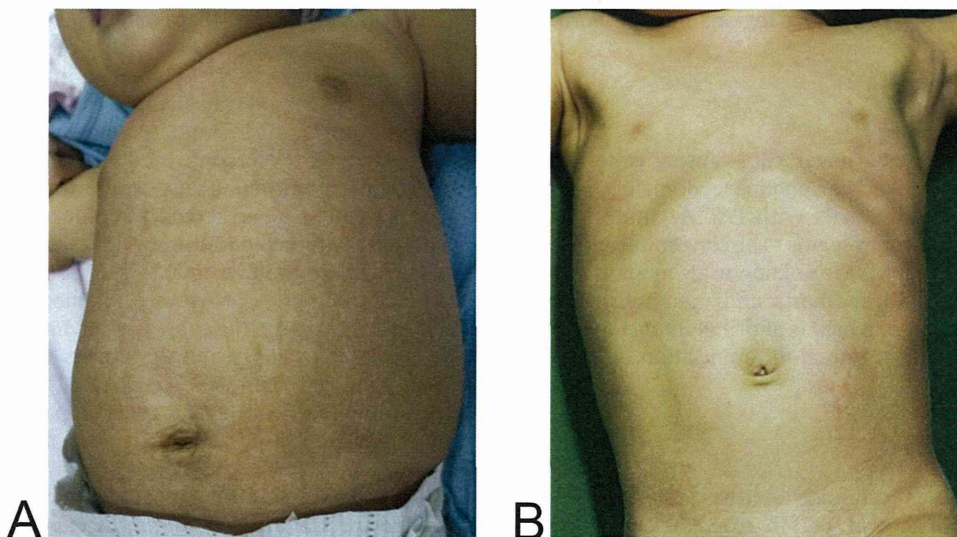

Fig. 4. Clinical features of mild LI with *TGM1* mutations and LI with *CYP4F22* mutations. Scales with slight partial erythema on the trunk of the patient are shown. The figures 4A and B are from Sugiura et al. [12] and Sugiura et al. [18], respectively. Scales on trunk of the patient (A) and scales with slight partial erythema on the trunk of the patient (B) are shown.

catalyzed by 12R-LOX/eLOX-3 constitutes an indispensable step for the covalent linkage of ω-hydroxyl ceramides to proteins of the cornified cell envelope and subsequently for the formation of the corneocyte lipid envelope. Furthermore, analysis of *Aloxe3*-knockout mice revealed a function of epidermal lipoxygenase-3 as a hepoxilin synthase and its pivotal role in barrier formation [35]. ARCI phenotypes caused by *ALOXE3* mutations are categorized as CIE, LI, or SHCB. LI patients with *ALOXE3* mutations have been identified in Europe, North Africa, the Middle East, and South Asia, and, recently, an LI patient with *ALOXE3* mutations was also reported in East Asia [15].

2.4. NIPAL4

Defects of NIPA-like domain-containing 4 (NIPAL4), encoded by NIPAL4, have also been linked to cases of LI or CIE phenotypes [16]. NIPAL4 has several transmembrane domains and belongs to a new family of proteins and its function is unknown. Li et al. [16] recently suggested that fatty acid transport protein 4 (FATP4), NIPAL4, and TGase-1 interact in lipid processing, which is essential for maintaining epidermal barrier function. It is also hypothesized that NIPAL4 serves as an Mg^{2+} transporter for FATP4 in this process [16]. FATP4 belongs to a family of six transmembrane proteins that facilitate long- and very long-chain fatty acid uptake [36]. FATP4 is expressed in several tissue types, including the skin. Mutations in human *SLC27A4*, which encodes FATP4, cause ichthyosis prematurity syndrome, characterized by a thick desquamating epidermis and premature birth [37].

2.5. CYP4F22

Mutations in *CYP4F22* have been identified as causative genetic defects in LI type 3 (OMIM #604777) [17]. *CYP4F22* encodes a cytochrome P450, family F polypeptide 2 homolog of leukotriene B4-omega-hydroxylase (CYP4F22). CYP4F22 is thought to be an orphan CYP. Its function has not yet been clarified, but CYP4F22 is highly expressed at the location and time corresponding to the onset of keratinization during skin development [38]. Patients with LI caused by *CYP4F22* mutations are very rare; only three reports cases have been reported in the literature. These reports describe homozygous mutations from consanguineous families in the Mediterranean region and Japan (Fig. 4B) [17,18]. Recently, we suggested possible genotype/phenotype correlations for *CYP4F22* mutations, i.e., a patient harboring one or two truncating CYP4F22 mutations affecting substrate-binding regions may be born as a collodion baby [18].

2.6. PNPLA1

PNPLA1 was identified as a novel ARCI-causing gene in 2012 [19]. It encodes patatin-like phospholipase domain protein 1 (PNPLA1). All affected individuals with *PNPLA1* mutations are born as CBs and the impairment seems to be congenital and severe at birth. The function of PNPLA1 is unknown, although it appears to play a key role in lipid synthesis and metabolism during epidermal barrier formation, which is of prime importance in the keratinization process and terminal differentiation of keratinocytes.

2.7. CERS3

CERS3, which encodes ceramide synthase 3 (CERS3), was identified as a causative gene of ARCI in 2013 [20,21]. Functional analysis of a skin sample and in vitro differentiated keratinocytes from a patient demonstrated that mutated CERS3 impairs the synthesis of ceramides with very long-chain acyl moieties [21].

2.8. LIPN

In affected members of a consanguineous Arab pedigree with a late-onset LI (#OMIN 613943), Israeli et al. [22] identified *LIPN* as a causative gene. *LIPN* encodes lipase N (LIPN). Although the function of LIPN has not been elucidated, it is thought to play a role in the differentiation of human keratinocytes [22].

3. Challenges related to the precise diagnosis of ARCI even with genetic tools

Dorfman–Chanarin syndrome (DCS) is a rare autosomal recessive form of congenital ichthyosis, characterized by the presence of intracellular lipid droplets in multiple organs [39,40] (OMIM #275630). Liver dysfunction and mental retardation are major extra-cutaneous manifestations of DCS [41,42]. DCS patients often have mutations in *ABHD5* (also termed as *CGI-58*), which encodes α/β hydrolase domain-containing 5 (ABHD5)/comparative gene identification 58 (CGI-58), an activator of adipose triglyceride lipase. ABHD5 deficiency leads to triglyceride accumulation [43,44]. We recently reported a case of DCS without mental retardation, caused by a homozygous *ABDH5* splicing site mutation [45]. We suggested that the *ABHD5* splicing site mutation, which results in the skipping of the entire exon 6, is associated with a DCS phenotype lacking mental retardation [45].

Takeichi et al. [46] have recently demonstrated the impact of next-generation sequencing technologies on clinical genetics in the dermatology field. Indeed, next-generation sequencing can reveal two autosomal recessive disorders in a single family. For example, in a patient showing an ARCI phenotype, mutations in both *ST14* and *POMT1*, which encode suppression of tumorigenicity 14 and O-mannosyltransferase, respectively, were detected by next-generation sequencing [47]. *ST14* is a causative gene for ARCI11 (OMIM #602400), known as ARCI with hypotrichosis, and follicular atrophoderma with hypotrichosis and hypohidrosis. *POMT1* is a causative gene for Walker–Warburg syndrome, a rare autosomal recessive disorder with developmental malformations.

4. mRNA analysis of hair samples to aid in genetic diagnosis

We hypothesized that mRNA expression in hair follicle epithelial cells is analogous to that of interfollicular epidermal cells. Accordingly, we developed an innovative method for skin epithelial mRNA analysis using RNA extracted from plucked hair samples to analyze mRNA expression in epithelial cells (keratinocytes). We confirmed that *ABCA12* mRNA expression in cutaneous epithelial cells can be analyzed using our method. That is, total RNA extracted from plucked hairs was successfully analyzed without performing invasive skin biopsies, to our knowledge, for the first time (Fig. 5) [8]. Total RNA was extracted from hair roots and cDNAs were obtained by reverse transcription-PCR. In an HI patient, the expression of *ABCA12* mRNA was evaluated by quantitative PCR using the cDNAs to estimate mRNA decay. Sequencing of the cDNA extracted from hair samples also demonstrated the aberrant splicing patterns due to splice-site mutations [8]. We recently applied our method using plucked hairs to analyze mRNA expression of other genes, including *IL36RN*, *ABHD5*, and *TRPS1*, in cutaneous epithelial cells [45,48–50]. Our method for the analysis of mRNA expression in epithelial cells (keratinocytes) using plucked hair samples can be applied to the diagnosis of not only keratinization disorders, but also many other genetic diseases in the dermatology field.
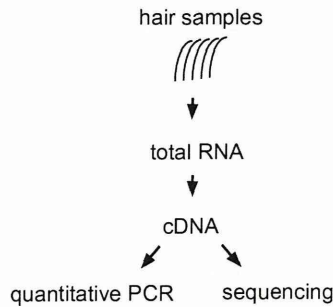
hair samples

total RNA

cDNA

quantitative PCR   sequencing

Fig. 5. Flowchart for the mRNA analysis of hair samples to aid in genetic diagnosis.

5. Concluding remarks

Knowledge of the molecular genetics and pathogenesis of ichthyosis has advanced dramatically. A genetic diagnosis method utilizing next-generation sequencing and analysis of mRNA using hair samples has been developed. In addition, the results of studies using disease models have suggested possible therapies for ARCI due to *ABCA12* or *TGM1* mutations. An increasing number of intensive studies on ARCI and causative molecules will promote efficient therapeutic methods for ARCI patients.

Funding

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References

[1] Oji V, Tadini G, Akiyama M, Blanchet Bardon C, Bodemer C, Bourrat E, et al. Revised nomenclature and classification of inherited ichthyoses: results of the First Ichthyosis Consensus Conference in Soreze 2009. J Am Acad Dermatol 2010;63:607–41.

[2] Russell LJ, DiGiovanna JJ, Rogers GR, Steinert PM, Hashem N, Compton JG, et al. Mutations in the gene for transglutaminase 1 in autosomal recessive lamellar ichthyosis. Nat Genet 1995;9:279–83.

[3] Huber M, Rettler I, Bernasconi K, Frenk E, Lavrijsen SP, Ponec M, et al. Mutations of keratinocyte transglutaminase in lamellar ichthyosis. Science 1995;267:525–8.

[4] Akiyama M. Harlequin ichthyosis and other autosomal recessive congenital ichthyoses: the underlying genetic defects and pathomechanisms. J Dermatol Sci 2006;42:83–9.

[5] Akiyama M, Sugiyama-Nakagiri Y, Sakai K, McMillan JR, Goto M, Arita K, et al. Mutations in lipid transporter ABCA12 in harlequin ichthyosis and functional recovery by corrective gene transfer. J Clin Invest 2005;115:1777–84.

[6] Kelsell DP, Norgett EE, Unsworth H, Teh MT, Cullup T, Mein CA, et al. Mutations in ABCA12 underlie the severe congenital skin disease harlequin ichthyosis. Am J Hum Genet 2005;76:794–803.

[7] Numata S, Teye K, Krol RP, Karashima T, Fukuda S, Matsuda M, et al. Mutation study for 9 genes in 23 unrelated patients with autosomal recessive congenital ichthyosis in Japan and Malaysia. J Dermatol Sci 2015.

[8] Takeichi T, Sugiura K, Matsuda K, Kono M, Akiyama M. Novel ABCA12 splice site deletion mutation and ABCA12 mRNA analysis of pulled hair samples in harlequin ichthyosis. J Dermatol Sci 2013;69:259–61.

[9] Shimizu Y, Sugiura K, Aoyama Y, Ogawa Y, Hitomi K, Iwatsuki K, et al. Novel ABCA12 missense mutation p.Phe2144Ser underlies congenital ichthyosiform erythroderma. J Dermatol 2013;40:581–2.

[10] Akiyama M, Sakai K, Hatamochi A, Yamazaki S, McMillan JR, Shimizu H. Novel compound heterozygous nonsense and missense ABCA12 mutations lead to nonbullous congenital ichthyosiform erythroderma. Br J Dermatol 2008;158: 864–7.

[11] Lefevre C, Audebert S, Jobard F, Bouadjar B, Lakhdar H, Boughdene-Stambouli O, et al. Mutations in the transporter ABCA12 are associated with lamellar ichthyosis type 2. Hum Mol Genet 2003;12:2369–78.

[12] Sugiura K, Suga Y, Akiyama M. Very mild lamellar ichthyosis with compound heterozygous TGM1 mutations including the novel missense mutation p.Leu693Phe. J Dermatol Sci 2013;72:197–9.

[13] Sugiura K. Unfolded protein response in keratinocytes: impact on normal and abnormal keratinization. J Dermatol Sci 2013;69:181–6.

[14] Jobard F, Lefevre C, Karaduman A, Blanchet-Bardon C, Emre S, Weissenbach J, et al. Lipoxygenase-3 (ALOXE3) and 12(R)-lipoxygenase (ALOX12B) are mutated in non-bullous congenital ichthyosiform erythroderma (NCIE) linked to chromosome 17p13.1. Hum Mol Genet 2002;11:107–13.

[15] Sugiura K, Akiyama M. Lamellar ichthyosis caused by a previously unreported homozygous ALOXE3 Mutation in East Asia. Acta Derm Venereol 2014.

[16] Li H, Vahlquist A, Torma H. Interactions between FATP4 and ichthyin in epidermal lipid processing may provide clues to the pathogenesis of autosomal recessive congenital ichthyosis. J Dermatol Sci 2013;69: 195–201.

[17] Lefevre C, Bouadjar B, Ferrand V, Tadini G, Megarbane A, Lathrop M, et al. Mutations in a new cytochrome P450 gene in lamellar ichthyosis type 3. Hum Mol Genet 2006;15:767–76.

[18] Sugiura K, Takeichi T, Tanahashi K, Ito Y, Kosho T, Saida K, et al. Lamellar ichthyosis in a collodion baby caused by CYP4F22 mutations in a non-consanguineous family outside the Mediterranean. J Dermatol Sci 2013;72:193–5.

[19] Grall A, Guaguere E, Planchais S, Grond S, Bourrat E, Hausser I, et al. PNPLA1 mutations cause autosomal recessive congenital ichthyosis in golden retriever dogs and humans. Nat Genet 2012;44:140–7.

[20] Radner FP, Marrakchi S, Kirchmeier P, Kim GJ, Ribierre F, Kamoun B, et al. Mutations in CERS3 cause autosomal recessive congenital ichthyosis in humans. PLoS Genet 2013;9:e1003536.

[21] Eckl KM, Tidhar R, Thiele H, Oji V, Hausser I, Brodesser S, et al. Impaired epidermal ceramide synthesis causes autosomal recessive congenital ichthyosis and reveals the importance of ceramide acyl chain length. J Invest Dermatol 2013;133:2202–11.

[22] Israeli S, Khamaysi Z, Fuchs-Telem D, Nousbeck J, Bergman R, Sarig O, et al. A mutation in LIPN, encoding epidermal lipase N, causes a late-onset form of autosomal-recessive congenital ichthyosis. Am J Hum Genet 2011;88: 482–7.

[23] Akiyama M. ABCA12 mutations and autosomal recessive congenital ichthyosis: a review of genotype/phenotype correlations and of pathogenetic concepts. Hum Mutat 2010;31:1090–6.

[24] Milstone LM, Choate KA. Improving outcomes for harlequin ichthyosis. J Am Acad Dermatol 2013;69:808–9.

[25] Shibata A, Ogawa Y, Sugiura K, Muro Y, Abe R, Suzuki T, et al. High survival rate of harlequin ichthyosis in Japan. J Am Acad Dermatol 2014;70:387–8.

[26] Shimizu Y, Ogawa Y, Sugiura K, Takeda J, Sakai-Sawada K, Yanagi T, et al. A palindromic motif in the −2084 to −2078 upstream region is essential for ABCA12 promoter function in cultured human keratinocytes. Sci Rep 2014;4:6737.

[27] Yanagi T, Akiyama M, Nishihara H, Ishikawa J, Sakai K, Miyamura Y, et al. Self-improvement of keratinocyte differentiation defects during skin maturation in ABCA12-deficient harlequin ichthyosis model mice. Am J Pathol 2010;177: 106–18.

[28] Cottle DL, Ursino GM, Ip SC, Jones LK, Ditommaso T, Hacking DF, et al. Fetal inhibition of inflammation improves disease phenotypes in harlequin ichthyosis. Hum Mol Genet 2015;24:436–49.

[29] Akiyama M, Shimizu H. An update on molecular aspects of the non-syndromic ichthyoses. Exp Dermatol 2008;17:373–82.

[30] Kim IG, McBride OW, Wang M, Kim SY, Idler WW, Steinert PM. Structure and organization of the human transglutaminase 1 gene. J Biol Chem 1992;267:7710–7.

[31] Akiyama M, Takizawa Y, Kokaji T, Shimizu H. Novel mutations of TGM1 in a child with congenital ichthyosiform erythroderma. Br J Dermatol 2001;144: 401–7.

[32] Vahlquist A, Bygum A, Ganemo A, Virtanen M, Hellstrom-Pigg M, Strauss G, et al. Genotypic and clinical spectrum of self-improving collodion ichthyosis: ALOX12B, ALOXE3, and TGM1 mutations in Scandinavian patients. J Invest Dermatol 2010;130:438–43.

[33] Tanahashi K, Sugiura K, Asagoe K, Aoyama Y, Iwatsuki K, Akiyama M. Novel TGM1 missense mutation p.Arg727Gln in a case of self-healing collodion baby. Acta Derm Venereol 2014;94:589–90.

[34] Aufenvenne K, Larcher F, Hausser I, Duarte B, Oji V, Nikolenko H, et al. Topical enzyme-replacement therapy restores transglutaminase 1 activity and corrects architecture of transglutaminase-1-deficient skin grafts. Am J Hum Genet 2013;93:620–30.

[35] Krieg P, Rosenberger S, de Juanes S, Latzko S, Hou J, Dick A, et al. Aloxe3 knockout mice reveal a function of epidermal lipoxygenase-3 as hepoxilin synthase and its pivotal role in barrier formation. J Invest Dermatol 2013;133:172–80.

[36] Schaffer JE, Lodish HF. Expression cloning and characterization of a novel adipocyte long chain fatty acid transport protein. Cell 1994;79:427–36.

[37] Klar J, Schweiger M, Zimmerman R, Zechner R, Li H, Torma H, et al. Mutations in the fatty acid transport protein 4 gene cause the ichthyosis prematurity syndrome. Am J Hum Genet 2009;85:248–53.

[38] Sasaki K, Akiyama M, Yanagi T, Sakai K, Miyamura Y, Sato M, et al. CYP4F22 is highly expressed at the site and timing of onset of keratinization during skin development. J Dermatol Sci 2012;65:156–8.

[39] Dorfman ML, Hershko C, Eisenberg S, Sagher F. Ichthyosiform dermatosis with systemic lipidosis. Arch Dermatol 1974;110:261–6.

[40] Chanarin I, Patel A, Slavin G, Wills EJ, Andrews TM, Stewart G. Neutral-lipid storage disease: a new disorder of lipid metabolism. Br Med J 1975;1:553–5.

[41] Akiyama M, Sakai K, Takayama C, Yanagi T, Yamanaka Y, McMillan JR, et al. CGI-58 is an alpha/beta-hydrolase within lipid transporting lamellar granules of differentiated keratinocytes. Am J Pathol 2008;173:1349–60.

[42] Ujihara M, Nakajima K, Yamamoto M, Teraishi M, Uchida Y, Akiyama M, et al. Epidermal triglyceride levels are correlated with severity of ichthyosis in Dorfman–Chanarin syndrome. J Dermatol Sci 2010;57:102–7.

[43] Lefevre C, Jobard F, Caux F, Bouadjar B, Karaduman A, Heilig R, et al. Mutations in CGI-58, the gene encoding a new protein of the esterase/lipase/thioesterase subfamily, in Chanarin–Dorfman syndrome. Am J Hum Genet 2001;69:1002–12.

[44] Akiyama M, Sawamura D, Nomura Y, Sugawara M, Shimizu H. Truncation of CGI-58 protein causes malformation of lamellar granules resulting in ichthyosis in Dorfman–Chanarin syndrome. J Invest Dermatol 2003;121:1029–34.

[45] Sugiura K, Suga Y, Akiyama M. Dorfman–Chanarin syndrome without mental retardation caused by a homozygous ABHD5 splice site mutation that skips exon 6. J Dermatol Sci 2014;75:199–201.

[46] Takeichi T, Nanda A, Liu L, Salam A, Campbell P, Fong K, et al. Impact of next generation sequencing on diagnostics in a genetic skin disease clinic. Exp Dermatol 2013;22:825–31.

[47] Takeichi T, Nanda A, Aristodemou S, McMillan JR, Lee J, Akiyama M, et al. Whole-exome sequencing diagnosis of two autosomal recessive disorders in one family. Br J Dermatol 2014.

[48] Sugiura K, Takemoto A, Yamaguchi M, Takahashi H, Shoda Y, Mitsuma T, et al. The majority of generalized pustular psoriasis without psoriasis vulgaris is caused by deficiency of interleukin-36 receptor antagonist. J Invest Dermatol 2013;133:2514–21.

[49] Sugiura K. The genetic background of generalized pustular psoriasis: IL36RN mutations and CARD14 gain-of-function variants. J Dermatol Sci 2014;74:187–92.

[50] Shibata A, Tanahashi K, Sugiura K, Akiyama M. TRPS1 haploinsufficiency results in increased STAT3 and SOX9 mRNA expression in hair follicles in trichorhinophalangeal syndrome. Acta Derm Venereol 2014.

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Essential role of the cytochrome P450 CYP4F22 in the production of acylceramide, the key lipid for skin permeability barrier formation

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A skin permeability barrier is essential for terrestrial animals, and its impairment causes several cutaneous disorders such as ichthyosis and atopic dermatitis. Although acylceramide is an important lipid for the skin permeability barrier, details of its production have yet to be determined, leaving the molecular mechanism of skin permeability barrier formation unclear. Here we identified the cytochrome P450 gene *CYP4F22* (cytochrome P450, family 4, subfamily F, polypeptide 22) as the long-sought fatty acid ω-hydroxylase gene required for acylceramide production. *CYP4F22* has been identified as one of the autosomal recessive congenital ichthyosis-causative genes. Ichthyosis-mutant proteins exhibited reduced enzyme activity, indicating correlation between activity and pathology. Furthermore, lipid analysis of a patient with ichthyosis showed a drastic decrease in acylceramide production. We determined that CYP4F22 was a type I membrane protein that locates in the endoplasmic reticulum (ER), suggesting that the ω-hydroxylation occurs on the cytoplasmic side of the ER. The preferred substrate of the CYP4F22 was fatty acids with a carbon chain length of 28 or more (≥C28). In conclusion, our findings demonstrate that CYP4F22 is an ultra-long-chain fatty acid ω-hydroxylase responsible for acylceramide production and provide important insights into the molecular mechanisms of skin permeability barrier formation. Furthermore, based on the results obtained here, we proposed a detailed reaction series for acylceramide production.

acylceramide | ceramide | lipid | skin | sphingolipid

A skin permeability barrier protects terrestrial animals from water loss from inside the body, penetration of external soluble materials, and infection by pathogenetic organisms. In the stratum corneum, the outermost cell layer of the epidermis, multiple lipid layers (lipid lamellae) play a pivotal function in barrier formation (Fig. S1) (1–3). Impairment of the skin permeability barrier leads to several cutaneous disorders, such as ichthyosis, atopic dermatitis, and infectious diseases.

The major components of the lipid lamellae are ceramide (the sphingolipid backbone), cholesterol, and free fatty acid (FA). In most tissues, ceramide consists of a long-chain base (LCB; usually sphingosine) and an amide-linked FA with a chain length of 16–24 (C16–C24) (4, 5). On the other hand, ceramide species in the epidermis are strikingly unique (Fig. S2A). For example, epidermal ceramides contain specialized LCBs (phytosphingosine and 6-hydroxysphingosine) and/or FAs with α- or ω-hydroxylation (1–3). In addition, substantial amounts of epidermal ceramides have ultra-long-chain FAs (ULCFAs) with chain lengths of 26 or more (≥C26) (4, 5). Unique epidermal ceramides are acylceramides having C28–C36 ULCFAs, which are ω-hydroxylated and esterified with linoleic acid [EOS in Fig. S1; EODS, EOS, EOP, and EOH in Fig. S2A; EOS stands for a combination of an esterified ω-hydroxy FA (EO) and sphingosine (S); DS, dihydrosphingosine; P, phytosphingosine; H, 6-hydroxysphingosine] (1–3, 6, 7). These characteristic molecules may be important to increase the hydrophobicity of lipid

lamellae and/or to stabilize the multiple lipid layers. Linoleic acid is one of the essential FAs, and its deficiency causes ichthyosis symptoms resulting from a failure to form normal acylceramide (8). Ichthyosis is a cutaneous disorder accompanied by dry, thickened, and scaly skin; it is caused by a barrier abnormality. In patients who have atopic dermatitis, both total ceramide levels and the chain length of ceramides are decreased, and ceramide composition is altered also (9–11).

In addition to its essential function in the formation of lipid lamellae, acylceramide also is important as a precursor of protein-bound ceramide, which functions to connect lipid lamellae and corneocytes (Fig. S1) (12, 13). After the removal of linoleic acid, the exposed ω-hydroxyl group of acylceramide is covalently bound to corneocyte proteins, forming a corneocyte lipid envelope. Acylceramides and protein-bound ceramides are important in epidermal barrier formation, and mutations in the genes involved in their synthesis, including the ceramide synthase *CERS3*, the 12(R)-lipoxygenase *ALOX12B*, and the epidermal lipoxygenase-3 *ALOXE3*, can cause nonsyndromic, autosomal recessive congenital ichthyosis (ARCI) (3, 14–16). CERS3 catalyzes the amide bond formation between an LCB and ULCFA, producing ULC-ceramide, which is the precursor of acylceramide (Fig. S1 and Fig. S2B) (17). ALOX12B and ALOXE3 are required for the formation of protein-bound ceramides (13, 18). Other ARCI genes include the ATP-binding cassette (ABC) transporter *ABCA12*, the transglutaminase *TGM1*, *NIPAL4* (NIPA-like domain containing

Significance

The sphingolipid backbone ceramide is the major lipid species in the stratum corneum and plays a pivotal function in skin permeability barrier formation. Acylceramide is an important epidermis-specific ceramide species. However, the details of acylceramide production, including its synthetic genes, reactions and their orders, and intracellular site for production, have remained unclear. In the present study, we identified the cytochrome P450, family 4, subfamily F, polypeptide 22 (CYP4F22) as the missing fatty acid ω-hydroxylase required for acylceramide synthesis. We also determined that CYP4F22 is a type I endoplasmic reticulum membrane protein and that its substrate is ultra-long-chain fatty acids. Our findings provide important insights into the molecular mechanisms of not only acylceramide production but also skin permeability barrier formation.

4)/*ICHTHYIN, CYP4F22*/*FLJ39501, LIPN* (lipase, family member N), and *PNPLA1* (patatin-like phospholipase domain containing 1) (16, 19). The exact functions of *NIPAL4, LIPN,* and *PNPLA1* are currently unclear. Causative genes of syndromic forms of ichthyosis also include a gene required for acylceramide synthesis: the FA elongase *ELOVL4,* which produces ULCFA-CoAs, the substrate of CERS3 (20).

Although acylceramide is essential for the epidermal barrier function, the mechanism behind acylceramide production is still poorly understood, leaving the molecular mechanisms behind epidermal barrier formation unclear. For example, acylceramide production requires ω-hydroxylation of the FA moiety of ceramide. However, the ω-hydroxylase responsible for this reaction was unidentified heretofore (Fig. S1). Here, we identified the cytochrome P450, family 4, subfamily F, polypeptide 22 (CYP4F22), also known as "FLJ39501," as this missing FA ω-hydroxylase required for acylceramide production. *CYP4F22* had been identified as one of the ARCI genes (21), although its function in epidermal barrier formation remained unsolved. Our findings clearly demonstrate a relationship between ARCI pathology, acylceramide levels, and ω-hydroxylase activity.

Results

Identification of CYP4F22 as the FA ω-Hydroxylase Required for ω-Hydroxyceramide Production.

Although researchers have long known that ω-hydroxylation is essential for acylceramide formation, they have puzzled over which gene is responsible for this reaction. To identify this gene, we first established a cell system that produced ULC-ceramides, a possible substrate of interest for ω-hydroxylase, because most cells cannot produce such extremely long ceramides. HEK 293T cells overproducing the FA elongase ELOVL4 and/or the ceramide synthase CERS3 were labeled with [^{3}H]sphingosine, and the chain lengths of ceramides were determined by reverse-phase TLC. Although overexpression of either ELOVL4 or CERS3 alone did not result in the production of ULC-ceramides, their co-overproduction caused generation of ULC-ceramides with $\geq$C26 (Fig. 1*A*). They migrated more slowly than long-chain (LC; C16–C20) ceramides and very long-chain (VLC; C22–C24) ceramides on reverse-phase TLC. Production of ULC-ceramides with chain lengths up to C36 also was confirmed by LC-MS analysis (Fig. S3). When labeled lipids were separated by normal-phase TLC, ULC-ceramides were detected as a band at the adjacent, upper position of VLC-ceramides (Fig. 1*B*).
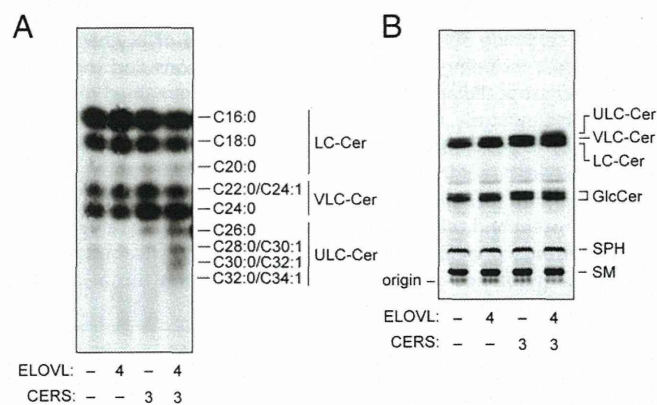

Fig. 1. Overproduction of ELOVL4 and CERS3 causes generation of ULC-ceramides. HEK 293T cells were transfected with plasmids encoding *3xFLAG-ELOVL4* and *3xFLAG-CERS3,* as indicated. Cells were labeled with [^{3}H]sphingosine for 4 h at 37 °C. Lipids were extracted, separated by reverse-phase TLC (*A*) or normal-phase TLC (*B*), and detected by autoradiography. Cer, ceramide; GlcCer, glucosylceramide; SM, sphingomyelin; SPH, sphingosine.
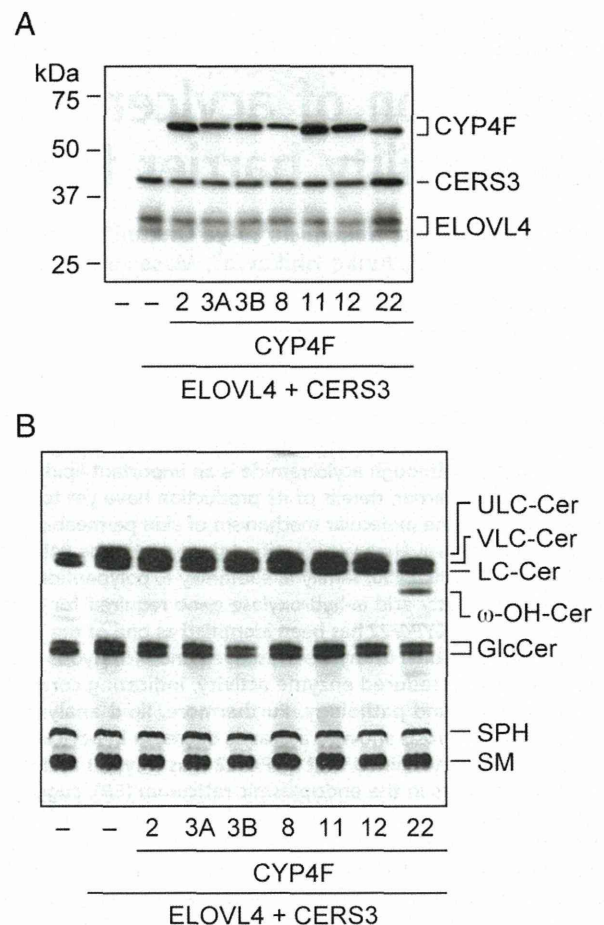

Fig. 2. CYP4F22 is involved in ω-hydroxyceramide synthesis. HEK 293T cells were transfected with plasmids encoding *3xFLAG-ELOVL4, 3xFLAG-CERS3,* and *3xFLAG-CYP4F* subfamily members, as indicated. (*A*) Total lysates prepared from the transfected cells were separated by SDS/PAGE, followed by immunoblotting with anti-FLAG antibodies. (*B*) Cells were labeled with [^{3}H]sphingosine for 4 h at 37 °C. Lipids were extracted, separated by normal-phase TLC, and detected by autoradiography. Cer, ceramide; GlcCer, glucosylceramide; SM, sphingomyelin; SPH, sphingosine; ω-OH, ω-hydroxy.

It has been reported that the cytochrome P450 (CYP) inhibitor aminobenzotriazole inhibits the generation of ω-hydroxyceramide in cultured human keratinocytes (22). In humans, 57 *CYP* genes exist, and mammalian *CYP* genes are classified into 18 families and 43 subfamilies. Some CYP4F members are implicated in the ω-hydroxylation of long-chain FAs (23, 24), raising the possibility that certain CYP4F subfamily members are responsible for ω-hydroxylation of ULCFAs in acylceramide formation. To test this possibility, we cloned all the human *CYP4F* subfamily genes (*CYP4F2, 3A, 3B, 8, 11, 12,* and *22*), and each was expressed as an N-terminally 3xFLAG-tagged protein in HEK 293T cells overproducing 3xFLAG-ELOVL4 and 3xFLAG-CERS3. All CYP4F subfamily proteins were expressed at similar levels (Fig. 2*A*). Among the CYP4F subfamily members, only CYP4F22 caused the disappearance of ULC-ceramide, which was concomitant with the production of a new band at the position of ω-hydroxyceramide (Fig. 2*B* and Fig. S4). LC-MS analysis determined that this band indeed represented ω-hydroxyceramides with C28–C36 (Fig. S5). Thus, CYP4F22 is the ω-hydroxylase required for ω-hydroxyceramide production.

Correlation Between CYP4F22 Activity and Ichthyosis Pathology.
Although the *CYP4F22* gene has been identified as one of the