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Figure Legends

Figure 1. ABCA12 protein structure and domains. Analysis of the ABCA12 predicted protein disclosed features that are typical of ABCA transporters.[1]

Figure 2. Immunofluorescence labeling using ultrathin cryosections as substrates revealed that glucosylceramide (green) and ABCA12 (red) overlapped in the granular layers (derived from Reference No. 5).

Figure 3. Scheme of ABCA12 distribution from cis-Golgi, trans-Golgi network to lamellar granules in the upper spinous and granular layer keratinocytes (derived from Reference No. 5).

Figure 4. Physiological role(s) of ABCA12 in lipid trafficking of epidermal keratinocytes and the model of pathogenetic mechanisms in ichthyosis phenotypes caused by ABCA12 deficiency. A: Model of how ABCA12 transports lipids in epidermal keratinocytes. B: Model of how loss of ABCA12 function leads to lipid abnormality and lipid barrier malformation in the upper epidermis. C: It is hypothesized that lipid barrier defects and disturbed keratinocyte differentiation coordinately cause hyperkeratosis and the ichthyosis phenotype (derived from Reference No. 20).

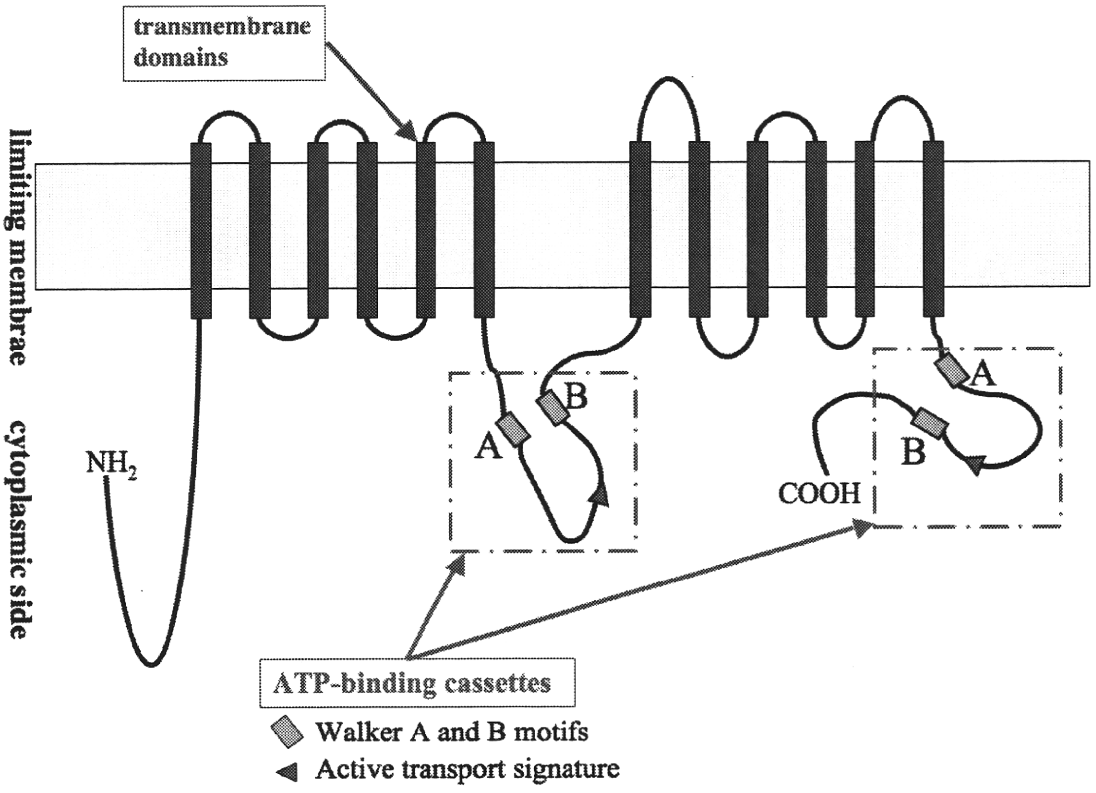

Figure 1.

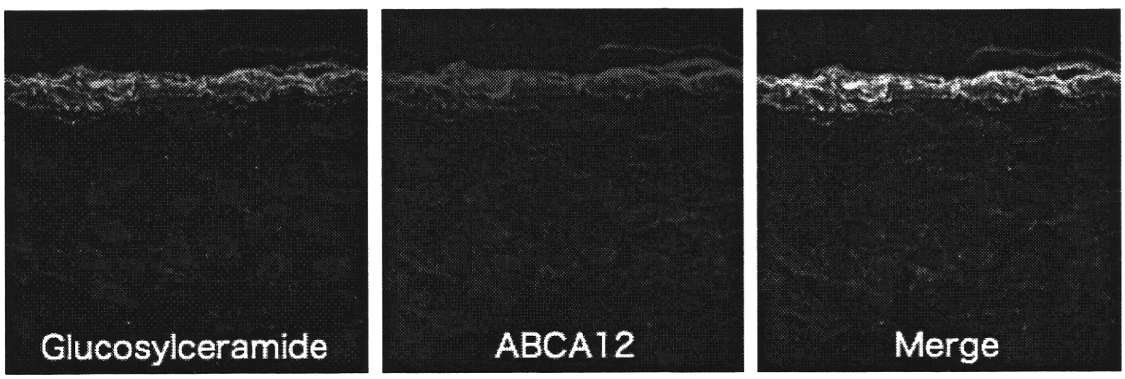

Figure 2.

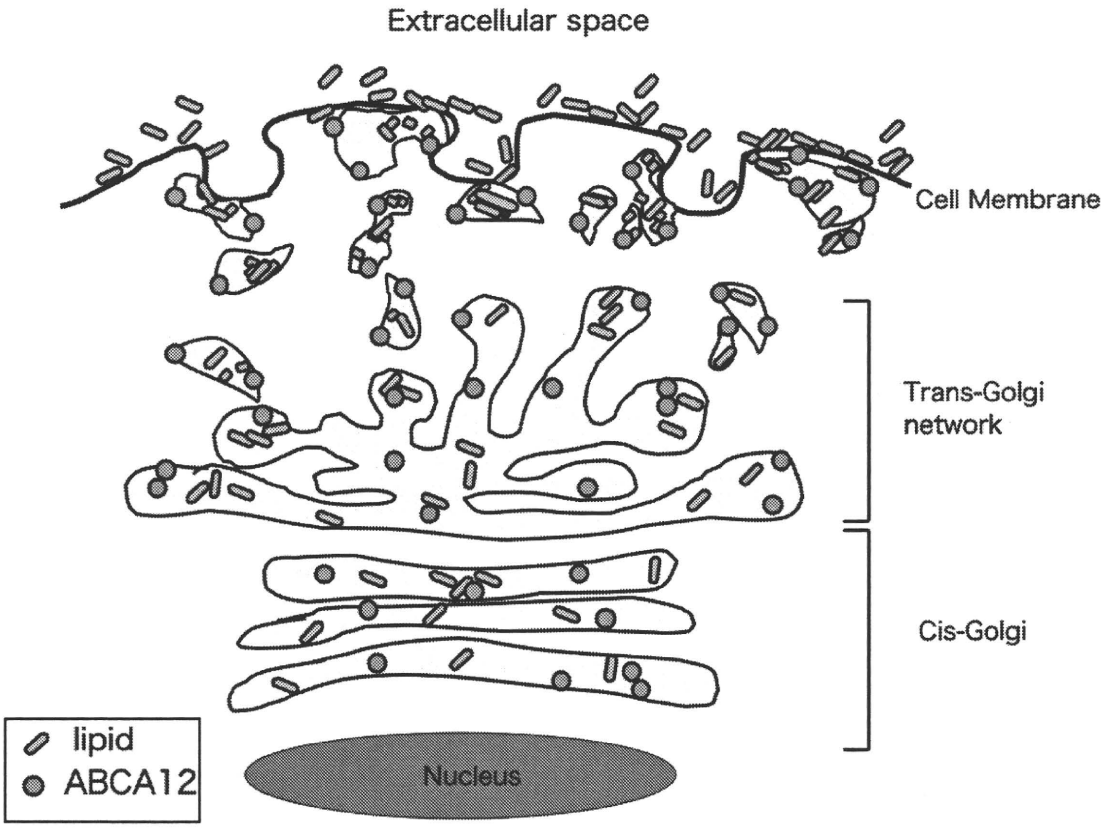

Figure 3.

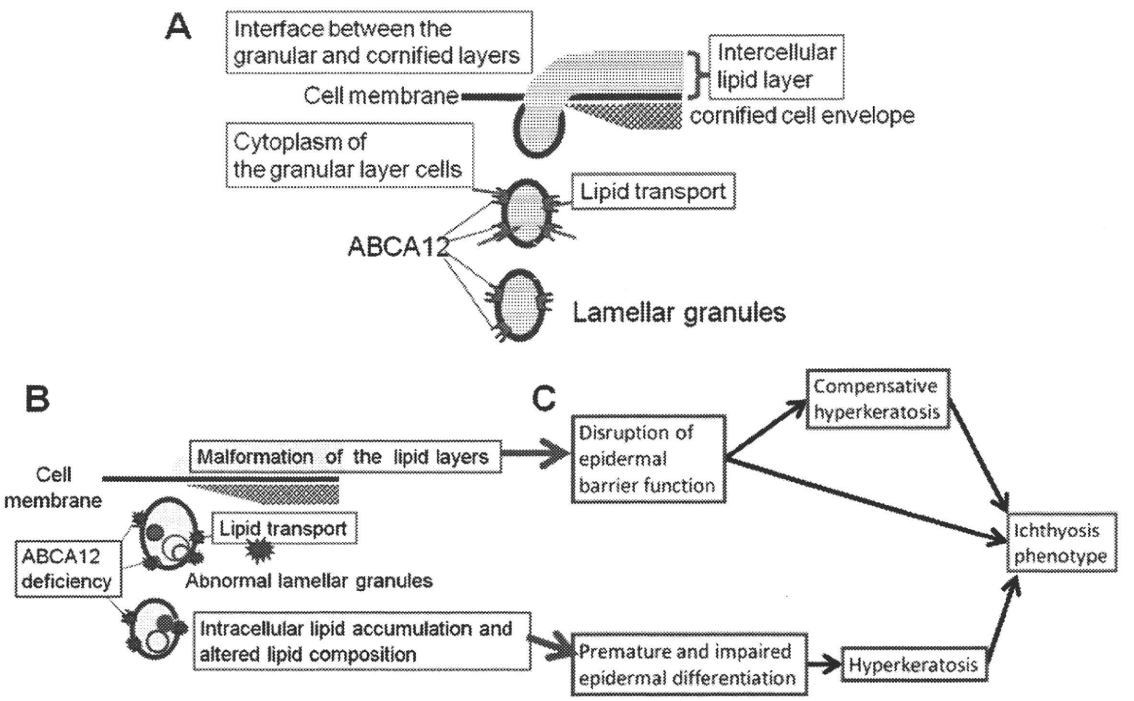

Figure 4.

Nagoya Journal of Medical Science

INVITED REVIEW ARTICLE

UPDATED MOLECULAR GENETICS AND PATHOGENESIS OF ICHTHYOSES

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ABSTRACT

Research into molecular genetics and the pathomechanisms of ichthyoses have considerably advanced and resulted in the identification of several causative genes and molecules underlying ichthyosis. In 2009, the First Ichthyosis Consensus Conference was held to establish a consensus for the nomenclature and classification of inherited ichthyoses and an international consensus for the classification of inherited ichthyosis has been achieved. In this review, the pathogeneses of various ichthyoses are summarized based on the revised classification and terminology of ichthyosis. Skin barrier defects are involved in the pathogenesis of various types of ichthyosis. The known causative molecules underlying ichthyosis include ABCA12, lipoxygenase-3, 12*R*-lipoxygenase, CYP4F2 homolog, ichthyin and steroid sulfatase, all of which are thought to be related to the intercellular lipid layers. ABCA12 is a known keratinocyte lipid transporter associated with lipid transport in lamellar granules and loss of ABCA12 function leads to defective lipid transport in the keratinocytes, resulting in the most severe, harlequin ichthyosis phenotype. Other causative molecules for ichthyoses are transglutaminase 1, keratins and filaggrin. Transglutaminase 1 has a function in cornified cell envelope formation. Keratins 1, 10 and 2 are involved in the keratin network of suprabasal keratinocytes and filaggrin are essential for formation of keratohyalin granules. It is important to obtain information concerning genetic defects and elucidate ichthyotic disease pathomechanisms for establishment of effective therapy and beneficial genetic counseling including prenatal diagnosis for families affected by ichthyotic disease.

Key words: ABCA12; epidermolytic ichthyosis; harlequin ichthyosis; lamellar ichthyosis; congenital ichthyosiform erythroderma; prenatal diagnosis

INTRODUCTION

The ichthyoses form a large, clinically and etiologically heterogeneous group of cornification disorders and typically affect all or most of the skin surface.[1] Six major distinct clinical subtypes are known in hereditary non-syndromic ichthyoses, i.e., starting with the most severe form: harlequin ichthyosis (HI, MIM#242500); lamellar ichthyosis (LI, MIM#242300); congenital ichthyosiform erythroderma (CIE, MIM#242100); epidermolytic ichthyosis (EI, MIM #113800); a mild and non-congenital form, recessive X-linked ichthyosis (RXLI, MIM#308100); to the mildest form of ichthyosis vulgaris (IV, MIM#146700).[2] Superficial epidermolytic ichthyosis (SEI, MIM #146800) is an additional subtype similar to EI. For a long time, the pathomechanisms and underlying genetic defects were unknown, although significant progress has recently been made in our understanding of the molecular basis of the human epidermal keratinization processes.

In 1978, the causative abnormality underlying RXLI was clarified as a steroid sulfatase deficiency caused by genetic defects in the steroid sulfatase gene (*STS*).[3,4] In 1992, mutations in the keratin 1 gene (*KRT1*) and keratin 10 gene (*KRT10*) were detected as a cause of EI.[5-7] Since transglutaminase (TGase) 1 gene (*TGM1*) mutations were identified as the cause in LI in 1995,[8,9] mutations in several other genes have also been identified in severe autosomal recessive congenital ichthyoses (ARCI).[10]

In 2005, loss of function mutations in the ABCA12 gene were reported to underlie HI, the most severe type of ichthyosis.[11,12] In 2006, null mutations in the gene coding filaggrin (*FLG*) were detected as the causative defects leading to IV.[13]

To date, the number of genes that have been identified and demonstrated to

cause ichthyosis in human patients, has reached eleven and these are shown in Table 1, i.e., *FLG*,[13] *KRT1, KRT10*,[5-7] *KRT2*,[14-16] *TGM1*,[8,9] *ABCA12*,[11,17,18] two lipoxygenase genes, *ALOXE3* and *ALOX12B*,[19] *NIPAL4*[20] and *FLJ39501*.[21] Majority of ichthyosis phenotypes mentioned above show primary abnormality associated with barrier function in the stratum corneum as their pathogenetic mechanisms.[1] The skin barrier of the stratum corneum has major three components, i.e., intercellular lipid layers, cornified cell envelope and keratin/filaggrin degradation products (Fig. 1)

In 2009, the First Ichthyosis Consensus Conference was held to establish a consensus for the nomenclature and classification of inherited ichthyoses and an international consensus for the classification of inherited ichthyosis has been achieved.[2] The new classification and nomenclature should be useful for all clinicians and can serve as a reference point for future research. In this updated review, based on the revised classification and terminology of ichthyoses, the pathogeneses of various ichthyoses are described in association with descriptions of specific defects in the essential components comprising the epidermal skin barrier, highlighting a few crucial diseases and mechanisms. In addition, I mentioned briefly the prenatal diagnosis of severe congenital ichthyosis at the end of the present review.

MAJOR ICHTHYOSIS SUBTYPES AND THEIR CAUSATIVE MOLECULES

Harlequin ichthyosis (HI)

Formation of the intercellular lipid layers is essential for epidermal barrier function and defective formation of the lipid layers is thought to result in serious loss of barrier function, and to extensive hyperkeratosis.[22] Formation of

the intercellular lipid layers is a highly complicated series of processes that include transport of lipids into the lamellar granules and multi-step metabolism of this lipid content within lamellar granules. ABCA12 has been highlighted, because ABCA12 was recognized as a key molecule in keratinocytes lipid transport.[11,23)]

Among the severe ARCI diseases, HI is the most devastating congenital ichthyosis and affected newborns show large, thick, plate-like scales over the whole body with severe ectropion, eclabium and flattened ears (Fig. 2A).[10)] In 2005, we revealed that ABCA12 is a keratinocyte lipid transporter and demonstrated that *ABCA12* mutations lead to the HI phenotype.[11)] Another group independently reported that *ABCA12* mutations underlie HI by linkage analysis.[12)] *ABCA12* mutations were reported to underlie LI and CIE cases.[17,18)] ABCA12 is a member of a large superfamily of the ATP-binding cassette (ABC) transporters, which bind and hydrolyze ATP to transport various molecules across a limiting membrane or into a vesicle.[24)] All ABCA subfamily members are thought to be lipid transporters.[25)] ABCA12 is a keratinocyte transmembrane lipid transporter protein associated with lipid transport in lamellar granules to the apical surface of granular layer keratinocytes.[11)]

Ultrastructurally, lamellar granule abnormalities are apparent in HI patient epidermis.[26)] Several morphologic abnormalities have been reported, for example abnormal lamellar granules in the granular layer keratinocytes and a lack of extracellular lipid lamellae in the stratum corneum, reflect defective lipid transport by lamellar granules and the malformation of intercellular lipid layers in the stratum corneum in HI.[26)] In addition, cultured epidermal keratinocytes from an HI patient carrying *ABCA12* mutations demonstrated defective glucosylceramide transport and this phenotype was recoverable by *in vitro ABCA12* corrective gene transfer.[11)] From these findings, we have shed

light on the pathomechanisms of HI with the underlying *ABCA12* mutations that leading to a loss of ABCA12 function. Mutations in the lipid transporter protein, ABCA12 cause defective lipid accumulation into lamellar granules which then become expelled from the apical surface of keratinocytes,[11,27] resulting in malformation of the intercellular lipid layers of the stratum corneum.[11] The fact that ABCA3 (a member of the same protein superfamily as ABCA12) functions in pulmonary surfactant lipid secretion again via the production of similar lamellar-type granules within lung alveolar type II cells[28,29] further supports this concept.

Subsequently,we transplanted cultured keratinocytes from patients with HI and succeeded in reconstituting HI skin lesions in immunodeficient mice.[27] These reconstructed HI lesions showed similar changes to those observed in HI patients' skin. In addition, we generated *Abca12* disrupted (*Abca12*$^{-/-}$) mice and our *Abca12*$^{-/-}$ mice closely reproduced the human HI phenotype, showing marked hyperkeratosis with eclabium and skin fissure.[30] Lamellar granule abnormalities and defective ceramide distribution were remarkable in the epidermis. Skin permeability assays of *Abca12*$^{-/-}$ mouse fetuses revealed severe skin barrier dysfunction after the initiation of keratinization. Another group independently developed *Abca12*$^{-/-}$ mice and the mice also confirmed the clinical features of HI[31] and a mouse strain carrying a homozygous spontaneous missense mutation was reported to show skin manifestations similar to HI.[32]

HI patients often die in the first one or two weeks of life. However, once they survive beyond the neonatal period, HI survivors' phenotypes improve within several weeks after birth. In order to clarify mechanisms of the phenotype recovery, we studied grafted skin and keratinocytes from *Abca12*-disrupted (*Abca12*$^{-/-}$) mouse and revealed that, during maturation, *Abca12*$^{-/-}$ epidermal

keratinocytes regain normal differentiation processes, although the exact mechanisms of this restoration is still unknown.[33)]

Congenital ichthyosiform erythroderma (CIE) and lamellar ichthyosis (LI)

Formation of a 15-nm-thick layer of protein on the inner surface of the cell membrane, called the cornified cell envelope (CCE) is essential for the skin barrier function.[1)] CCE is assembled by accumulation of several precursor proteins including involucrin, small proline-rich proteins and loricrin.[34)] TGases in the epidermis are thought to be responsible at least in part, for the assembly of cornified cell envelope precursor proteins to form the cornified cell envelope.[35)] TGase 1, the major subtype of the three TGases expressed in the epidermis,[36,37)] is a membrane-associated TGase of about 92 kD. Since the identification of TGase 1 gene (*TGM1*) mutations in a number of families with LI in 1995,[8,9)] additional *TGM1* mutations have been reported in LI families. In addition, *TGM1* mutations were reported to underlie CIE phenotype.[38,39)]

It is of little doubt that defective formation of stratum corneum intercellular lipid layers is caused by abnormal keratinocyte lipid metabolism, transport, and/or secretion as one of the major pathogenetic mechanisms underlying congenital ichthyosis. Several critical molecules causing ichthyosis are thought to be involved in the formation of the stratum corneum intercellular lipid layers.[1)]

In 2003, a keratinocyte lipid transporter *ABCA12* was reported as the causative gene in type 2 LI (OMIM #601277) (see "harlequin ichthyosis" section above).[17)] Type 2 LI is a subtype of LI which links to 2q33-35. Several genotype/phenotype correlations with *ABCA12* mutations have been elucidated as follows.[40)] Combinations of missense mutations resulting in only one amino acid alteration underlie the LI phenotype.[17)] In contrast, most mutations in HI

are truncation or deletion mutations which lead to more severe changes such as loss of function of ABCA12 peptide affecting important nucleotide-binding fold domains and/or transmembrane domains. In HI, so far at least one mutation on each allele must be a truncation or deletion mutation within a conserved region that seriously affects ABCA12 function.[11,40,41] In the Japanese population, CIE patients harboring *ABCA12* mutations as the causative genetic defect are not rare (Fig. 2B).[18,42,43] Further accumulation of data on *ABCA12* mutations and their effects on protein function/structure together with specific mutation sites is needed to better elucidate genotype/phenotype correlations and help predict HI patient prognosis.

In 2002, mutations in two lipoxygenase genes, *ALOXE3* and *ALOX12B*, coding lipoxygenase-3 and 12(R)-lipoxygenase, respectively were reported to underlie ARCI.[19] Lipoxygenase-3 and 12(R)-lipoxygenase are non-heme iron-containing dioxygenases expressed in the epidermis, and their exact functions are unknown.[44,45] They may be associated with lipid metabolism of the lamellar granule contents and/or intercellular lipid layers in the epidermis. 12(R)-lipoxygenase knockout mice demonstrated a severe impairment of skin barrier function.[46] The loss of barrier function was associated with perturbance of the assembly/extrusion of lamellar granules. Cornified cell envelope from skin of 12(R)-lipoxygenase deficient mice showed increased fragility.[46] Lipid analysis demonstrated a disordered composition of ceramides, especially a decrease of ester-bound ceramide species.[46] From these findings, the 12(R)-lipoxygenase-lipoxygenase-3 pathway was thought to play a key role in the process of epidermal barrier formation by affecting lipid metabolism.[46] In fact, partially disturbed lamellar granule secretion was reported in the epidermis of a CIE patient with *ALOX12B* mutations (Akiyama et al, 2010).[47]

NIPAL4 (ichthyin) defects were also reported to underlie certain cases of LI or

CIE phenotype.[20] NIPAL4 is a protein with several transmembrane domains, which belongs to a new family of proteins with an unknown function. NIPAL4-like proteins are localized in the plasma membrane, and share homologies to both transporters and G-protein coupled receptors.[20] NIPAL4 was suggested to be a membrane receptor for certain ligands (trioxilins A3 and B3) from the hepoxilin pathway,[20] although the exact mechanisms of how *NIPAL4* mutations lead to an ichthyotic phenotype remain to be clarified.

Mutations in *FLJ39501* were identified in lamellar ichthyosis type 3 (MIM 604777) as causative genetic defects.[21] *FLJ39501* encodes a cytochrome P450, family 4, subfamily F, polypeptide 2 homolog of the leukotriene B4-omega-hydroxylase (CYP4F2). The exact function of CYP4F22 has not been elucidated yet, but it is thought to catalyze the 20-hydroxylation of trioxilin A3 form the 12(R)-lipoxygenase pathway. Further oxidation of this substrate would lead to 20-carboxy-(R)–trioxilin A3. This compound is suspected to be involved in skin hydration and would be an essential product missing in various forms of ARCI.

Epidermolytic ichthyosis (EI) and superficial epidermolytic ichthyosis (SEI)

A normal keratin filament network is an important structure for keratohyalin granule formation and for maintaining the integrity and dimensions of the cornified cell cytoplasm. In this context, the keratin-network is essential for normal skin barrier formation. Mutations in differentiation-specific keratins are known to result in ichthyosis phenotypes.

EI is caused by mutations in either the keratin 1 gene (*KRT1*) or keratin 10 gene (*KRT10*).[5-7] Most of the causative mutations are missense mutations that reside within the beginning or at the end of the rod domain segments of the keratin peptides,

which are called helix initiation and helix termination motifs. The helix boundary motifs are highly conserved regions of approximately 20 amino acids, which have been implicated in molecular overlapping interactions as part of the formation of 10 nm intermediate filaments from dimers comprising both type I acidic and a type II basic-neutral keratins.[48] Single amino acid alterations in these essential, helix boundary motifs frequently lead to a significant disease phenotype in the majority of the keratin diseases.

EI is a severe congenital ichthyosis which exhibits widespread blisters and erosions on a background of erythrodermic skin from birth.[1,2] After the perinatal period, blister formation ceases and generalized hyperkeratosis becomes apparent. Histologically, predominant vacuolization of the cells is observed in the middle and upper spinous layers and granular layers of the epidermis. The vacuolated keratinocytes have large and irregularly shaped cytoplasmic granules. Ultrastructurally, irregularly shaped, abnormal, clumped keratin filaments are seen in the keratinocytes of the upper spinous layer to the granular layer.[49] EI generally shows autosomal dominant inheritance, although only a few families showing recessive inheritance trait have been reported.[50,51] In such families with recessive inheritance, causative mutations are nonsense mutations. As for genotype-phenotype correlations in EI, palmoplantar keratoderma exists in patients with *KRT1* mutations, but not in patients with *KRT10* mutations.[52]

SEI is also an autosomal dominant ichthyosis which shows similar, but slightly milder clinical features than those of EI.[53] Keratin 2 gene (*KRT2*) mutations underlie SEI patients.[14-16] Occasionally, cases with SEI can be difficult to clinically differentiate from EI and molecular genetic studies are essential for a more definite diagnosis.[54] In the human epidermis, keratins 1 and 10 expression occurs in the suprabasal layers, replacing keratin 5 and keratin 14 as cells differentiate. Keratin 2 is expressed somewhat later than keratins 1 and 10 in

keratinocyte differentiation as keratinocytes approach the granular layer. Thus, consistent with the restricted keratin 2 expression sites, in SEI, clumped keratin filaments were restricted to the cytoplasm of granular layer cells and the uppermost spinous layer cells, leading to granular degeneration only in he uppermost spinous and granular layers of the patient's epidermis.
The restricted granular degeneration results in the milder clinical manifestations and the presence of superficial denuded areas (the *mauserung* phenomenon) which are characteristic to SEI.

Ichthyosis vulgaris (IV)

IV is a common genetic keratinization disorder, clinically characterized by scaling, especially on the flexor limbs, and with palmoplantar hyperlinearity. The epidermis of IV patients shows a decrease in the size and number or, complete absence of keratohyalin granules.[55)]

Degradation products of keratohyalin granules occupy the cytoplasm of keratinized cells in the stratum corneum and play important roles in the skin barrier function. Keratohyalin granules in the granular layer of the epidermis are predominantly composed of large >400-kDa, profilaggrin polyproteins.[56,57)] Upon terminal differentiation of keratinocytes, profilaggrin is cleaved into 10-12 essentially identical 37-kDa filaggrin peptides. The liberated filaggrin aggregates the keratin filaments,[56)] causing the collapse of the granular cells into flattened squame-shape. In addtion, degradation products of filaggrin contribute to moisture retention in the cornified layers. Thus, filaggrin, a major component of keratohyalin granules, is indispensable for the normal, intact skin barrier function. In this context, loss or reduction of filaggrin expression results in excessively dry skin and impaired barrier function, which leads to clinical features of IV.

In 2006, *FLG* mutations were identified in IV patients in the European populations[13] and have been shown to be major predisposing factors for atopic dermatitis.[58] Subsequently, *FLG* mutations were identified in Japanese, Chinese, Taiwanese and Korean populations.[59,60] Based on the information of population-specific *FLG* mutations, numbers of cohort studies of atopic dermatitis for *FLG* mutations have been performed and about 25-50% of patients with atopic dermatitis were demonstrated to harbor *FLG* mutations as a predisposing factor. It was demonstrated that *FLG* mutations are strongly associated with atopic dermatitis also in the Japanese population.[59,61] Skin barrier defects due to *FLG* mutations are thought to play important roles in pathogenesis of atopic diseases including atopic dermatitis, allergic rhinitis and asthma.[62]

Recessive X-linked ichthyosis (RXLI)

Genetic defects in the steroid sulfatase gene (*STS*) was reported to underlie RXLI.[3,4] Most *STS* mutations underlying RXLI are large deletions and, nowadays, fluorescence *in situ* hybridization (FISH) techniques is a useful tool to detect causative *STS* mutations.[63] The hyperkeratosis and scaling in RXLI is associated with abnormal accumulation of cholesterol sulfate in the stratum corneum.[64] Steroid sulfatase is concentrated in lamellar granules and then secreted into the intercellular space of stratum corneum, along with other lamellar granule-derived lipid hydrolases.[65] In the intercellular space of stratum corneum, steroid sulfatase degrades cholesterol sulfate, generating some cholesterol for the barrier. Furthermore, the progressive decline in cholesterol sulfate permits corneodesmosome degradation leading to intact desquamation.[65] Thus, two molecular pathways contribute to disease pathogenesis in RXLI. Steroid sulfatase deficiency leads both to malformation of the intercellular lipid barrier, and delay of corneodesmosome degradation, resulting in corneocyte retension.[65] In addition, increased Ca^{2+} in the

intercellular space of the stratum corneum in X-linked ichthyosis was reported to contribute to corneocyte retention, by increasing corneodesmosomes and interlamellar cohesion.[65)]

PRENATAL DIAGNOSIS OF SEVERE CONGENITAL ICHTHYOSES

The quality of life of patients with severe congenital ichthyoses is seriously affected in some cases and the parents' request for prenatal diagnosis cannot be easily ignored. Due to the recent advances in our understanding of the genetic defects underlying severe congenital ichthyosis, it has become possible to make DNA-based prenatal diagnosis for congenital ichthyosis families by chorionic villus or amniotic fluid sampling in the earlier stages of pregnancy with a lower risk to fetal health and with a reduced burden on the mothers compared with prenatal diagnosis by fetal skin biopsy.[1)]

In the case of HI, before identification of the causative gene *ABCA12*, prenatal diagnosis had been performed by fetal skin biopsy and electron microscopic observation at the later stages of pregnancies, 19-23 weeks estimated gestational age, for more than 20 years.[66-69)] When a fetus was diagnosed as affected, it was a serious problem to perform interruption of the pregnancy at the late stage of pregnancy.

After identification of *ABCA12* as the causative gene for HI, it has become feasible to perform DNA-based prenatal diagnosis for HI by chorionic villus or amniotic fluid sampling at a much earlier stage of pregnancy, with a significantly lower risk to fetal health and a reduced burden on mothers, as in the case of other severe genetic disorders.[70)] Indeed, prenatal diagnosis and exclusion of HI by DNA testing were performed in our laboratory.[70,71)]

Prenatal diagnosis of LI by ultrastructural observation of fetal skin samples is at